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6. AUTHOR(S)
Claudio M. Aldaz, M.D., Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
The University of Texas M.D. Anderson Cancer Center
Houston, Texas 77030

E-MAIL:
maldaz@odin.mdacc.tmc.edu

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The main hypothesis of this project was that breast cancer cells utilize various alternative mechanisms to circumvent a major restriction point in the G1 phase of cell cycle progression. Furthermore, we speculated that the sum of the various subsets of tumors with abnormalities in either player of the cell cycle inhibitor *p16* pathway, accounts for a very significant number of breast cancers. It was a main objective of this research project to elucidate the overall extent of *p16* involvement in the tumorigenesis of the breast. An additional major goal was to evaluate whether *p16* fulfills all the criteria to be considered a senescence control gene. In the course of these studies we determined that 1) Abnormalities affecting the INK4A locus are common in breast cancer; 2) Disregulation of expression of the genes encoded by such locus (p16 and $p14^{ARF}$) affects most breast carcinomas. Overexpression of p16 associates with more aggressive breast cancer frequently, while many other breast carinomas show loss of p16 expressing; and 3) p16 inactivation is a key necessary event that HMEC use to achieve extended life *in vitro* and to overcome the M0 senescence arrest.

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PRINCIPAL INVESTIGATOR: Claudio M. Aldaz, M.D.

CONTRACTING ORGANIZATION: The University of Texas M.D.
Anderson Cancer Center
Houston, Texas 77030

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1. INTRODUCTION

The $p16^{INK4a}$ protein, hereafter referred to as p16, is a known negative regulator of cell cycle progression through its cyclin dependent kinase (CDK) inhibiting function (1). p16 competes with the activating D type cyclins for association with CDK4 or CDK6, thereby preventing phosphorylation of proteins controlling G1 exit such as the retinoblastoma (Rb) protein (2). Inactivation of *p16* has been observed in numerous tumor types (3-8) and lack or reduced expression of *p16* has also been shown through a variety of technical approaches to occur in at least 50% of the breast cancer samples examined (9, 10). While these findings suggest that *p16* may play an important role in breast tumorigenesis, the consequence of such aberrations of *p16* are not yet clear.

Of the putative functions of *p16*, the role as a candidate senescence gene is supported by several observations. The mapping of *p16* to chromosomal subregion 9p21, a region containing a putative senescence gene, is significant in this regard (11). Also, p16 is an upstream negative regulator of retinoblastoma protein phosphorylation (1). Observations from various laboratories also suggested that *p16* may be important in the control of replicative senescence.

Our original hypothesis was that breast cancer cells utilize various alternatives mechanisms to circumvent a major restriction in the G1 phase of the cell cycle. Furthermore we speculated that the sum of the various subsets of tumors with abnormalities at various levels of the p16 pathway, accounts for a very significant number of breast cancers. The main objective of this research project was to elucidate the overall extent of p16 involvement in the tumorigenesis of the breast. An additional major goal was to evaluate whether p16 fulfills al the criteria to be considered a senescence control gene.

2.BODY

2.1. Evidence of p16 Involvement in Breast Cancer

Initial studies by Kamb et al. have originally shown p16 to be homozygously deleted in 60% of breast carcinoma lines(12). This high rate of p16 deletion implicated p16 to be a tumor suppressor whose inactivation is involved in breast carcinogenesis. Because of this we determined to first ascertain the involvement of p16 in breast carcinogenesis in vivo. When 24 primary breast carcinomas were analyzed by PCR amplification of polymorphic microsatellite markers surrounding p16, LOH or allelic imbalance of markers from this region was observed in approximately 58% of cases. These results from our laboratory were reported in a **first manuscript (13)** which addressed Task 1 of the proposal. This findings per se were novel since the chromosome area 9p21 had not previously reported to be involved in previous studies. In order to determine if the remaining p16 allele was affected, as predicted by two-hit Knudson's hypothesis as well as previous examples dictated by other tumor suppressors, we subjected the same tumors analyzed for LOH to p16 mutational analysis. Single-strand conformational polymorphism analysis o p16 exon 1 and direct sequencing of exon 2 was performed on a series of 21 primary breast carcinomas. Of the 21 tumors analyzed only 1 showed a mutation of probable consequence (13). So the high frequency of LOH without corresponding mutational inactivation of the remaining allele was intriguing and justified to conduct further investigations.

Various reports also suggested that mutation may not be the primary mechanism of inactivation of the p16 gene in many tumor types (3-6). To further complicate the interpretation of results it was also discovered at that time, that an alternative transcript was encoded from the same second and third exons of p16, but utilizing a separate promoter and alternative first exon, referred as exon 1 β (14, 15). This alternative transcript is translated from an alternative reading frame to that of p16 and results in a protein of 14kd (p14ARF) in humans and 19kd (p19ARF) in mice (16). Thus, the *p16* locus is complex with two overlapping transcripts translated from distinct reading frames, resulting in two polypeptides,

$p16^{INK4a}$ and $p19^{ARF}$, each able to induce cell cycle arrest. Because the transcripts of these two polypeptides partially overlap, it may be that alterations which affect one, may also affect the other. Therefore, the possibility exists of $p19^{ARF}$ being a protein with tumor suppressive function being targeted for inactivation as well. So we decided to perform a comprehensive analysis of the aberrations affecting the *p16* and p19 genes and expression of transcripts *in vivo* in order to help to clarify these issues.

To help elucidate the role of aberrations affecting these two genes in breast cancer, we performed a comprehensive analysis of inactivation and expression in a series of primary breast carcinomas. To that end we used interphase chromosomal fluorescence *in situ* hybridization (IC-FISH) deletion analysis of the *p16* region, methylation analysis of the 5' region of the primary first exon, SSCP analysis of the alternative exon 1β transcript, and expression of both transcripts (α and ß) by semi-quantitative RT-PCR. Taken together with previously determined microsatellite polymorphism LOH analysis of 9p21 and *p16* mutational analysis in these same tumors (13), we believe we obtained a more complete account of $p16^{INK4a}$ and putative $p19^{ARF}$ involvement in breast tumorigenesis.

These result have been reported in a **second publication**, Brenner et al. (9) which addressed Task 2 of the original application. A summary of Results and Discussion of this manuscript read as follows.

2.1.a. $p16^{INK4a}$ Locus Copy Number.

In order to determine if these tumors may have alternatively incurred homozygous deletion of the *p16* region, and to what extent, we performed dual color interphase chromosomal fluorescence *in situ* hybridization using the 250Kb *p16* cos cosmid contig and a centromeric probe for chromosome 9 copy number. We observed total or partial deletion of the *p16* chromosomal region in 61% (11 of 18) of breast tumors. This result is comparable to our aforementioned analysis of the same tumor set by PCR based microsatellite length polymorphism analysis (13). Specifically, three cases (17% of total) displayed tumor cell subpopulations with total deletion of the p16 cos region. Subsequent analysis by RT-PCR showed low to absent expression in both of the two cases for which sufficient material was available. However, while partial deletion was more common (11 cases) than total deletion, subsequent expression analysis did not show a strong association between partial deletion and loss of expression. Tumor cell populations displaying hyperdiploidy of chromosome 9 were observed in eight (45%) of the tumors analyzed. No tumors showed evidence of significant chromosome 9 monosomy.

To our knowledge, this was the first report of homozygous deletion of the *p16* region in primary breast carcinomas through the use of *in situ* hybridization.

2.1.b. Hypermethylation of $p16^{INK4a}$ Exon 1 α.

Exon 1 of the $p16^{INK4a}$ gene contains a documented CpG island which has been shown to be unmethylated in normal tissue and hypermethylated in certain tumor types at varying incidence (3-5). In order to establish the methylation status of the 5' region, total genomic DNA was digested with a combination of a flanking site endonuclease and a methylation sensitive endonuclease, as previously described (5). Twenty three tumors were analyzed, of which patterns consistent with partial or total methylation were observed in four (17%). Two tumors (T30 and T41) showed methylation with multiple restriction enzymes (SacII and SmaI), while two others (T16 and T44) revealed methylation with only one enzyme (EagI and SacII, respectively). Of those tumors showing methylation, three (T16,30,41) displayed patterns consistent with methylation of all possible endonuclease sites in that region, while one displayed methylation of a single site (T44). The remaining 19 tumors revealed no pattern consistent with hypermethylation. These results were consistent with a previous report of methylation in primary tumors of the breast, although the frequency observed here (17%) is somewhat lower than the frequency previously reported (31%) by Herman et. al. (5).

2.1.c. Expression of $p16^{INK4a}$ Alpha and Beta Transcripts.

As previously suggested by Stone et al., the similarity in size and sequence of the α and ß transcripts may have complicated previous efforts to measure *p16* RNA levels by Northern blot (14) in different neoplasias. Only an analysis of transcripts using the unique sequences of exon 1α would be able to asses the true levels of *p16* expression. Additionally, since inactivating events that target *p16* may also affect the alternative β transcript, and since we know the alternative β transcript to encode $p19^{ARF}$ and have growth suppressive effects in murine cells *in vitro*, it would be advantageous to evaluate both *p16* and alternative β transcript expression independent of one another.

Expression level of *p16* α and β transcripts in twenty three tumors was determined by RT-PCR analysis. Expression levels of each transcript were subsequently compared to mean normal expression of a panel of four normal breast samples. Expression of both the *p16* primary α and alternative β transcripts in breast tumors was varied. Six of twenty three (26%) showed expression of $p16^{INK4a}$ at levels less than 10% of normal mean, while another 5 (22%) revealed levels of expression from 10% to 30% of normal mean (i.e. greater than 70% reduction in normal expression). Loss of expression in many of these tumors can be accounted for by either hypermethylation (T16, T30, and T41) or homozygous deletion (T6 and T28). However, inactivation by either of these mechanisms was not observed in some cases in which loss of expression was observed, indicating that other modes of inactivation could be operative. Moreover, a previous report of $p16^{INK4a}$ expression by immunohistochemistry suggested loss of expression in as much as 65% of breast tumors (10), indicating that inactivating events might be possible at a post-transcriptional stage as well. We also observed that two additional carcinomas, T2 and T54, displayed what appears to be overexpression of $p16^{INK4a}$, concomitant with β overexpression. Previous analyses in numerous lines had indicated that the overexpression of *p16* can be associated with Retinoblastoma protein (pRB) inactivation (17). However, no precedent of this association had been described *in vivo*. Nonetheless, such overexpression may be deemed aberrant.

Analysis of β transcripts showed great variability in expression, with apparent overexpression to be as prevalent as lack of expression. One of the twenty three tumors analyzed revealed undetectable levels of expression (T16). Two additional carcinomas (T6 and T26) showed expression below 30% of the normal mean. Incidentally, the two cases in which the lowest expression of β was observed (T6 and T16), both displayed α loss, and by distinct mechanisms. Loss of expression in tumor T6 appeared to be through homozygous deletion, while loss of expression in tumor T16 appeared to be through methylation and LOH (13). However, methylation of exon 1 α only explains loss of expression of the primary α transcript. Perhaps in some cases, such as tumor T16, the methylation of the 5' region of exon 1α is indicative of the hypermethylation of the entire locus, and as such, the 5' region of exon 1β also could be hypermethylated. Since the 5' region of exon 1ß from -180 bp to +266 bp contains a 70% GC content, and a CG:GC ratio of 0.71, thus defining a CpG island, this probability exists. Of additional interest, four tumors (T2, T20, T41, and T54) showed considerably high levels of β ($p19^{ARF}$) expression between 3 to 5 fold greater than the normal mean. What level of increased expression can be considered significant, and the possible implications of such overexpression, have yet to be determined.

The possibility of $p19^{ARF}$ being tumor suppressive in function as well has been previously demonstrated (14, 16). However, previous attempts to address this issue through sequence analysis of exon 1 β in other tumor types revealed no mutations (15). In this report, we addressed the issue of possible β inactivation in breast cancer by performing SSCP analysis of the exon 1 region of β transcripts in all twenty three tumors for which we obtained expression data, and found no evidence of mutation in any of the tumors (data not shown). While our own previous analysis (13) of exon 2 in breast tumors revealed three mutations of 21 tumors affecting amino acid sequence for the β transcript (CGA-GGA, codon 87; GCA-GTA, codon 96; CGC-CAC, codon 161), only one of these mutations was

found in a region conserved in both mouse and human, and another was a frequently reported polymorphism. Further, we have now shown that apparent loss of expression of the β ($p19^{ARF}$) transcript is primarily observed in those breast tumors in which $p16^{INK4a}$ expression is compromised. Taken together, this information indicates that point mutation or loss of expression is not common for the β transcript, and that there is no evidence to suggest a tumor suppressive role for the β transcript in breast carcinogenesis. However, it is unclear the reason for, and possible consequences of, the observed overexpression of the β transcript in some breast tumors. Further experiments are needed to address these issues.

Nevertheless, comprehensive analysis of homozygous and hemizygous deletion, methylation, mutation, and expression suggest that the tumor suppressor $p16^{INK4a}$ is cumulatively affected in approximately 40-50% of the breast carcinomas analyzed. This rate of inactivation of $p16^{INK4a}$, and lack of inactivation of the β transcript, implicate $p16^{INK4a}$ involvement in the tumorigenesis of the breast at a rate greater or equal to that previously reported for any other tumor suppressor gene in sporadic breast cancer.

2.2. Evidence of p16 Role in Senescence Control of Mammary Cells.

In our first p16 related manuscript (13) we observed that *p16* was found inactive in immortal breast epithelial lines derived from normal mammary epithelium through three distinct mechanisms; homozygous deletion (MCF10 and MCF12 cell lines) nonsense mutation with hemizygous loss (184 A1 cell line), or hypermethylation (184 B5 cell line). These findings supported p16 inactivation as a possible necessary event for escaping senescence. We sought to further characterize the role of *p16* in senescence control of human mammary cells. To this end normal primary human mammary epithelial cultures (HMEC) were followed from initial propagation *in vitro* until growth arrest and subsequent extended replicative growth. Expression analysis of *p16*, as well as another candidate senescence control gene $p21^{WAF1}$, was performed at various time points. The HMEC were also monitored for replicative capacity and expression of the senescence associated β-galactosidase. These results were reported in a **third publication** Brenner et al. (18).

A summary of the Results and Discussion of this manuscript reads as follows:

2.2.a Analysis of *p16* in Normal HMEC.

Normal HMEC *in vitro*, like normal fibroblasts, undergo a limited number of cell divisions (19). In the case of HMEC, the total number of population doublings which can be achieved, as well as other characteristics, has been shown to be dependent upon the type of media used to propagate the cells (19). Specifically, finite lifespan HMEC grown in the serum containing medium, MM, display active growth for 2-5 passages, or 15-25 population doublings, with gradual loss of proliferative activity. The senescent population retains the typical epithelial morphology (20). In contrast, when HMEC are grown in the serum-free MCDB 170 medium, after active proliferation for 2-3 passages, almost all the cells cease growth, becoming large, flat, striated, with irregular cell borders (20, 21). Following 2-4 weeks of inactivity, the population then undergoes a process termed "self-selection" (21). There is active proliferation of small cells with the typical epithelial cobblestone morphology, which soon dominate the culture. These post selection cells maintain growth for an additional 7-24 passages (approximately 45-100 population doublings in total), after which flatter and more vacuolated cells appear which retain the epithelial morphology without further growth arrest.

To assess the possible role of p16 in HMEC senescence, HMEC from five different reduction mammoplasty specimens were cultured in both MM and MCDB 170 and followed from initial propagation through cessation of growth. Initially, the levels of *p16* were low in both culture conditions.

These levels rose with increasing passage until initial growth arrest, when *p16* levels were 10-25 fold higher than original values. In contrast, assay of the emergent post-selection cells in MCDB 170 indicated that the level of *p16* transcript was drastically reduced. None of the post-selection HMEC revealed expression of *p16* during the following growth period, nor at the final growth arrest. These findings were confirmed at the protein level as well by immunoblotting analysis of the 184 HMEC culture using an anti-p16 antibody. Cells grown in serum containing media (MM) start with low levels of p16 protein expression which increases and remains high until final growth arrest. On the other hand and as observed with the p16 transcript analyses, when HMEC are grown in serum-free MCDB170 medium, p16 protein is detected in the pre-selection cells but not in the post-selection cell population (see attached manuscript Brenner, 98) (18).

2.2.b. Methylation of Post-Selection HMEC.

Previous analysis of tumor cell lines as well as numerous primary tumors has shown inactivation of *p16* through de novo methylation of the promoter region (5). Promoters silenced by methylation can be reactivated in many cases by treatment with 5-aza-2'-deoxycytidine, a drug which is an established inhibitor of DNA methylation. In order to determine whether cells arising after initial arrest in MCDB 170 lacked expression due to methylation, post selection cells were grown in the presence of 5-aza-2-deoxycytidine and evaluated for *p16* expression. Of the 5 post-selection cultures examined, all regained *p16* expression following treatment. Further, analysis of the 184 post-selection cells by methylation-sensitive endonuclease digestion of DNA followed by Southern hybridization with a *p16* exon 1 probe, showed patterns consistent with complete methylation of *p16*, corroborating the aforementioned results (not shown). These data indicate that those cells capable of post-selection growth were inactive in *p16* expression due to de novo methylation.

2.2.c. Coincidence of Expression of p16 and Senescence Associated β-galactosidase.

In analysis of senescent cultures, Dimri et al. (22) observed the expression of an unusual endogenous β-galactosidase with a pH optimum of 6.0 This form of β-galactosidase was expressed by senescent cells *in vivo* as well as those cultured *in vitro*, but not by presenescent, quiescent, or terminally differentiated cells. While the source or function of this novel β-galactosidase is unknown, it nevertheless constitutes a useful senescence associated marker. To establish whether this marker showed coincident expression with growth arrest and increased *p16* expression of the HMEC grown in both culture conditions, cells were evaluated at various time points for the presence of senescence-associated β-galactosidase (SA β-Gal).

As expected, analysis of SA β-Gal in the early actively growing HMEC in either MM or MCDB 170 revealed no activity. As previously mentioned, these same cell populations also showed low *p16* expression. However, when cells ceased replicating following the 2^{nd}-5^{th} passage in both media, high levels of SA β-Gal were displayed, coincident with the highest levels of *p16* . In contrast, the newly emergent post-selection HMEC from the same cultures in MCDB 170, which had no *p16* expression due to apparent hypermethylation of the *p16* promoter, did not display SA β-Gal activity positive cells. When these actively growing cells were treated with 5-aza-2-deoxycytidine to reverse methylation, cells ceased growing, regained expression of *p16* (as mentioned above), and expression of SA β-Gal was again observed concurrently (see attached manuscript Brenner 98) (18).

2.2.d. Analysis of $p21^{WAF1}$ Expression with Senescence.

Another CDK inhibitor, $p21^{WAF1}$, has been shown to be induced during senescence in fibroblasts (23, 24). In order to determine whether *p21* expression is associated with senescence of HMEC, levels of *p21* expression were followed in the aforementioned cultures. High levels of *p21* were observed during initial propagation, and showed little variability during growth arrest or extended replicative life.

Thus, while increased expression of *p21* was seen with senescence in some cell types, this does not appear to be the case with HMEC, suggesting that *p21* does not play as significant a role in senescence of HMEC as $p16^{INK4a}$. This is similar to observations in adrenocortical cells, which express high levels of $p21^{WAF1}$ throughout their replicative life span to senescence (25).

2.2.e. Discussion from Brenner et al. 98

As previously mentioned, the number of population doublings which can be achieved in the culture of HMEC has been shown to be largely dependent upon the type of culture media used (19). Nevertheless, all HMEC undergo a period of initial growth arrest at an approximately similar level of growth, from which only those cells grown in serum free media recover. The reason cells undergo this initial growth arrest and the mechanisms by which cells recover from it, have not been understood. In the series of HMEC examined in this report, all showed an increase in *p16* expression with progression toward initial growth arrest, at which point the highest levels of *p16* were observed. When grown in serum free media, foci of actively growing cells emerged from these seemingly senescent cultures at a low frequency (~10^{-5}), and were devoid of *p16* expression due to possible hypermethylation of the *p16* promoter region. These cells did not regain *p16* expression at anytime during their remaining replicative period, nor at final growth arrest. These data would suggest that increased expression of p16 may be causative of the initial growth arrest observed in these cultures.

It has been suggested (26) that initial growth arrest of HMEC may be indicative of a normal senescence and an early mortality stage which is distinct from of those previously postulated in the Shay et al. (27) two stage model of human mammary epithelial immortalization. Indeed, the increased expression of *p16* in fibroblasts with onset of senescence has been previously described (23), and we now show a similar increase with the onset of initial growth arrest in HMEC. Additionally, our finding of expression of senescence marker SA β-Gal in cells at this initial growth arrest, as well as in those post selection cells growth arrested with regain of p16 expression when treated with a methylation inhibitor, adds weight to this prospect. Nevertheless, while SA β-Gal may be the best marker available for determination of senescence, it is not an absolute indicator. Further, SA β-Gal was also observed during the final growth arrest which has classically been viewed as senescence. Additional studies are needed to better address how the initial arrest and final arrest may relate to senescence *in vivo*.

As previously mentioned, analysis of immortalization of human mammary epithelial cells by E6 and E7 genes has shown susceptibilities distinct to those found in fibroblasts (28). Therefore, it has been proposed that while both Rb and p53 are involved in fibroblast immortalization, only p53 appeared to play a role in mammary epithelial cells (27, 28). However, it is worth noting that the analysis of susceptibility to immortilization by E6 and E7 was performed with "post-selection" mammary epithelial cells grown in media MCDB 170, which we have now shown to lack *p16* expression (28). This would suggest that the lack of necessity for E7 in immortalization of mammary epithelial cultures is due to prior inactivation of *p16*, and subsequent alteration of the Rb pathway. In such circumstances, the sequestration of Rb protein by E7 may confer no known additional benefit. Further, it suggests that similar to fibroblasts, both Rb and p53 dependent pathways play a role in the senescence control of mammary epithelial cells.

In support of this argument, recent studies by Wazer et al. (29) have revealed that mammary epithelial cells present in milk and grown in a high serum supplemented media, similar to cells grown in serum supplemented media MM which we show to maintain *p16* expression, show substantial extension of life span with E7. While E6 was not able to confer a similar extension of life span in these cultures, its presence was required for complete immortalization. Additionally, in similar analysis of susceptibility to E6 and E7 immortalization of pre-selection and post-selection mammary epithelial cultures from tissue grown in media DFCI-1, a medium of constitution similar to MCDB 170 which results in selection, Wazer et al. (29) were able to demonstrate that most pre-selection HMEC cultures

undergo extension of lifespan followed by immortalization with E7 and not E6. In contrast, post-selection HMEC immortalize with E6 and not E7. This was subsequently corroborated by others, and the arrest which E7 is capable of circumventing was designated M0 by Foster and Galloway (26) to conform to nomenclature previously used in a two stage model of immortalization suggested by Shay et al. (28). Thus, the requirement for E7 in immortalization of cells expressing *p16* (pre-selection), and lack of necessity for E7 in the immortalization of mammary epithelial cells with inactivated *p16* (post-selection), supports the role of *p16* as a senescence control gene in human mammary epithelial cells. Additionally, this suggests that a chronology exists in the senescence control of human mammary epithelial cells where increased p16 expression may be involved in an initial stage (M0) of normal growth arrest. Inactivation of this initial control stage (e.g. p16 inactivation or Rb sequestration by E7) appears to result in a limited increase in replicative capacity and susceptibility to further extension of lifespan by circumvention of the second (M1) stage (e.g. by E6 or mutant p53) (28). Following a final period of selection (crisis, M2) immortal cells emerge. This may be typified by previous experiments such as those of spontaneous immortalization of post-selection (grown in MCDB 170) mammary epithelial cells from Li-Fraumeni patients, while fibroblasts from these same patients required E7 to immortalize (30). Adaptation of previously suggested two stage models of mammary epithelial cell immortalization to include a first step dependent upon Rb pathway inactivation might be necessary.

2.3. Evidence of a Role for p16 in Growth Suppression of Mammary Cells.

As described in the previous section, substantial evidence has been reported which implicates a role for *p16* loss in the escape from replicative senescence. To understand whether such loss of expression in mammary epithelial cells may have resulted in the escape from replicative senescence suggested by preliminary findings in other cell types, we performed analysis of *p16* expression in immortal and normal mammary epithelial cultures. Loss of *p16* was observed in all 4 immortal lines studied, as well as in cells in normal primary mammary epithelial cultures which were able to grow beyond the typical replicative life span, as previously discussed.

In order to further substantiate the role of *p16* in replicative senescence, we transfected a constitutive eukaryotic expression vector into one immortal mammary epithelial cell line in which loss of *p16* was observed. Further, to address whether abrogation of the Rb dependent pathway in which p16 functions was sufficient to induce extended replicative capacity, we transfected a normal primary mammary epithelial culture with an expression vector with a CDK4 mutant insert bearing a mutation in the p16 binding domain. These studies discussed here are only preliminary and have not been published. Nevertheless, they provide useful information for the design of future experiments which may further substantiate and elucidate the role of *p16* loss in breast tumorigenesis.

2.3.a. p16 Transfection of MCF-10.

MCF-10F cultures were plated 24 hours before transfection and grown to 70% confluency in four 60 mM dishes. Plasmid DNA (10 μg) of the empty pRcCMV vector and vector containing the p16 cDNA insert were combined with 25 μl of Transfectam (Promega) in a final volume of 2 ml of serum free media each. Each DNA lipid complex (1mL/dish) was immediately added in duplicate to washed cells with 0.5mL of media, left in contact overnight, and replaced with complete media. Seventy two hours following transfection, cells were trypsinized and plated at a density of 10,000 cells per dish in complete media containing G418 at a final concentration of 150 μg/ml. Three weeks following transfection, dishes were fixed in 10% neutral buffered formalin and stained with Geimsa. Transfections for isolation of inducible clones were as described above, except utilizing the pMAM vector (Clonetec) with the p16 cDNA insert in sense and antisense orientation, and vector alone.

CDK4-R24C Transfections. Cells from culture B24 in growth media MM were electroporated at 300V with a pulse length of approximately 24 msec in complete MM media containing 5 x 106 cells per ml and 10 μg of pHOOK vector (Invitrogen, San Diego, CA) containing either no insert or the CDK4-R24C cDNA, in a final volume of 0.4 ml per cuvette. Nine cuvettes were used per vector. Selection was performed following 72 hours utilizing 150 μg per ml of Zeocin (Invitrogen).

2.3.b. Preliminary Results and Discussion

Induction of Arrest by p16. In order to help characterize p16's growth suppressive abilities in immortal breast cells lacking p16, we transfected a constitutive mammalian expression vector carrying the full length p16 cDNA into MCF-10F. Upon selection of transfected cells in a colony forming assay, we observed near complete growth suppression of the transfected cells. Upon further examination of the dishes transfected with the p16 insert, we observed that although very few large colonies were formed, after two weeks of G418 selection, it was still possible to observe live cells and abortive colonies with a phenotype compatible with senescence. In contrast, control vector only transfectants displayed vary numerous large actively growing colonies.

Extended life of HMEC by Abrogation of p16 Function. A cyclin-dependent kinase 4 mutant (CDK4 R24C) was originally identified as a tumor-specific antigen recognized by HLA-A2.1-restricted autologous cytolytic T lymphocytes (CTLs) in a human melanoma. The mutation was part of the CDK4 peptide recognized by CTLs, prevented binding and inhibition by $p16^{INK4a}$, but not of $p21^{WAF1}$ or of $p27^{KIP1}$, nor did it affect the kinase activity. In essence, this CDK4 mutation subverted cell cycle arrest through selectively impaired interaction with p16. This same mutation was also identified in some familial melanoma kindreds (31). If p16 plays a significant role in HMEC cellular senescence through a CDK4 dependent pathway, as suggested by p16 expression and Rb phosphorylation states in senescent HMEC, then cells expressing this CDK4 mutant should be impaired in their ability to senesce, analogous to cells lacking p16 expression.

In order to further substantiate the role of p16 inhibition of Rb phosphorylation in replicative senescence, normal HMEC grown in growth medium MM were transfected with a construct containing the CDK4 R24C cDNA sequence in a constitutive eukaryotic expression vector (pHOOK). While the normal HMEC senesced at PDL 12.5, a stable clone was isolated which was able to grow beyond this normal life span. An additional 25 population doublings have been achieved to date, and cells continue to grow. Control transfectants containing the empty vector alone remained viable, yet ceased growth at a level equal to that of untransfected cells. However, it must be noted that these results are based upon isolation of only a single clone. Since these results could be related to the integration of the vector or a random mutation, further experiments are necessary for validation, although no extended growth was seen in control transfectants or untransfected primary cultures.

The high degree of difficulty in transfecting normal human mammary epithelial cultures is well known. Nevertheless, the results reported in this chapter are encouraging. It would appear to be necessary to use a more efficient viral mediated approach.

2.4. Studies of Multiple Cell Cycle Genes Breast Cancer Samples.

Our described observations substantiate a role for p16 inactivation in the tumorigenesis of the breast and as a target of 9p allelic loss. Interestingly, however some breast tumors showed overexpression of p16 indicating that involvement of this gene as well as that of $p14^{ARF}$ (homolog of mouse $p19^{ARF}$), encoded at the same locus in an alternative reading frame is more complex than previously thought. Thus our findings and hypothesis have to be re-interpreted and reviewed in light of a better understanding of the involved cell cycle players in breast cancer.

Of the cell cycle proteins, the Rb protein, cyclin D1, cyclin E, p16 and p27 have all been observed to be affected in breast carcinogenesis. Cyclin D1 has been shown to be both amplified in 10-20% of breast tumors and overexpressed in the majority of breast tumors (32-34). Cyclin D1 competes with p16 for binding with the CDKs. When cyclin D1 is more abundant than p16, it binds to and activates CDK4 and CDK6. Cyclin D1 mRNA and estrogen receptor expression were found to be positively correlated in primary breast cancer (35). There is no conclusive evidence however demonstrating that estrogen receptor directly up regulates cyclin D1 transcription. Other studies have suggested that overexpression of cyclin E, which is often found in breast tumors, is functionally redundant to cyclin D (36). In cells overexpressing both cyclin E and p16, cyclin E can functionally replace cyclin D providing tumor cells with a growth advantage (36). They do this by activating CDK's which in turn phosphorylate Rb, releasing E2F and initiating gene transcription, leading to cell cycle progression and a self-perpetuating positive regulatory loop. Interestingly, it was recently reported a bad prognosis and very high mortality rate in women with breast cancers showing high cyclin E expression concomitant with low expression of the CDK inhibitor $p27^{kip1}$ (37).

Inactivation of Rb itself has been described in breast cancer as a means of enhancing cell cycle progression. Although, when multiple modes of inactivation are accounted for, Rb is inactivated in less then 20% of breast cancers (38, 39). In the vast majority of tumor lines there is an inverse relationship between Rb and p16 expression (40, 41). (i.e., breast tumor cell lines which retain Rb expression have no expression of p16. Whereas, cell lines retaining p16 expression often lack expression of Rb.) We discussed above our findings with p16 in breast cancer.

Positive detection of the p53 protein accumulation has been shown to be associated with p53 mutations and a higher risk of breast cancer recurrence (42). Therefore, p53 inactivation appears to be a critical event in the tumorigenesis of the breast. This also suggests that an additional consequence of p53 inactivation would be abrogation of cell cycle arrest through loss of transcriptional activation of p21.

Interestingly, recently it was demonstrated an important regulatory link between both the P53 and Rb pathways. At the center of this link is the recently described $p14^{ARF}$ (previously called $p19^{ARF}$ because of the mouse homolog). As mentioned above, this gene is encoded at the INK4a locus in chromosome 9p21, as an alternative reading frame of the cyclin dependent kinase inhibitor $p16^{INK4a}$. It was demonstrated that the putative tumor suppressor ARF gene physically interacts with MDM2 and as a consequence basically blocks MDM2-induced p53 degradation and transcriptional inactivation (43, 44). Thus, this interaction leads to increase p53 stability and accumulation. Further strengthening the connection between the p53 and Rb pathways, it was very recently reported that ARF is transcriptionally upregulated by E2F1 (45). This allowed to speculate that perhaps abnormal cell proliferation, which results in E2F1 increase, in turn would result in increase of ARF which would lead to cell arrest or apoptosis via p53. This would not happen if an additional abnormality takes place such as p53 mutation or ARF inactivation (45). In studies from our laboratory we have demonstrated that both ARF and p16 expression levels are highly variable in breast cancer (9). We observed subsets of tumors that lack expression of both genes (p16 and ARF) due to common inactivation events such as homozygous deletion. On the other hand we have also observed numerous tumors that dramatically overexpressed ARF. We are currently addressing the p53 status in the same tumors.

We have performed a multiparametric analysis of the various cell cycle in a series of breast cancer tumors, studies which addressed Task 3 of the original application. Preliminary results on a set of approximately 80 breast carcinomas have been reported (Aldaz, et al Proceedings 1998 AACR). We are currently in the process of including larger number of cases in the analysis in order to achieve further statistical significance. Among the interesting findings we observed that low p16 levels are significantly associated with tumors that are positive for detection of Cyclin D1. On the other hand tumors overexpressing p16 were negative for Cyclin D1 expression. Interestingly these tumors with high p16

were also negative for progesterone receptor expression. These same tumors have a tendency to be estrogen receptor negative and lymph node positive for metastases and with high S fraction. Rb inactivation appears not to be the reason for high p16 since these breast tumors were found to be positive for Rb expression. This information suggests that tumors with p16 overexpression and Cyclin D1 negative may represent a more advance stage of progression perhaps unresponsive to the growth suppressive effects of p16. On the other hand tumors with low p16 expression levels and positive for Cyclin D1 expression may represent an earlier stage of tumor development.

KEY RESEARCH ACCOMPLISHMENTS

- We demonstrated that Loss of heterozygosity affecting the INK4A ($p16/p14^{arf}$) locus is a common event in breast cancer.
- We provided evidence that p16 inactivation occurs in some breast cancers via diverse mechanisms such as homozygous deletion and promoter methylation.
- We demonstrated that p16 expression is highly variable in breast cancer.
- We provided evidence for the first time that p16 is deleted or inactive immortal cells derived from "normal" mammary epithelium.
- We demonstrated that p16 methylation is a necessary event for normal mammary cells to achieve extended life in culture. Thus implicating p16 as major player in senescence control.
- Overexpression of p16 appears to correlate with more aggressive cancer phenotypes.

REPORTABLE OUTCOMES

Publications:

Brenner, A. J. and Aldaz, C. M. Chromosome 9p allelic loss and *p16/CDKN2* in breast cancer and evidence of *p16* inactivation in immortal breast epithelial cells. Cancer Res., *55:* 2892-2895, 1995.

Brenner, A.J., Paladugu, A., Wang, H., Olopade, O.I., Dreyling, M.H., and Aldaz, C.M. Preferential loss of expression of $p16^{INK4a}$ rather than $p19^{ARF}$ in breast cancer. Clinical Cancer Res. *2*: 1993-1998, 1996.

Brenner, A.J., Stampfer, M.R, and Aldaz, C.M. Increased *p16* expression with first senescence arrest in human mammary epithelial cells and extended growth capacity with *p16* inactivation. Oncogene 17: 199-205, 1998.

Charpentier, A., and Aldaz, C.M. The Molecular Basis of Breast Carcinogenesis. In: Molecular Basis of Human Cancer: Genomic Instability and Molecular Mutation in Neoplastic Transformation. W.B. Coleman and G.J. Tsongalis (eds.) NJ, The Humana Press Inc. in press.

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Bednarek, A.K., Brenner, A., and Aldaz, C.M. Identification of the human mammary epithelial cell subpopulation expression telomerase. Proc. Amer. Assoc. Cancer Res., *40*: 1759, 1999.

Degrees Obtained:

Andrew J. Brenner conducted many of the experiments and the research described was the topic of his doctoral thesis which allowed him to obtain the Ph.D. degree from the University of Texas at Austin in 1997.

Personnel:

C. Marcelo Aldaz, M.D., Ph.D.
Andrew Brenner, Ph.D.
Abhaya Paladugu, Ph.D.
Kendra Laflin, B.A.

CONCLUSIONS

Abnormalities affecting the INK4A locus are common in breast cancer. Disregulation of expression of the genes encoded by such locus (p16 and $p14^{ARF}$) affects most breast carcinomas. Overexpression of p16 associates with more aggressive breast cancer frequently. On the other hand, p16 inactivation is a key necessary event that human mammary epithelial cells use to achieve extended life in vitro and to overcome the so called M0 senescence arrest. These results support *p16* as well as the Rb pathway in the senescence control of normal mammary epithelial cells.

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APPENDICES

C. Marcelo Aldaz, M.D., Ph.D.

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Final Report

[CANCER RESEARCH 55, 2892–2895, July 1, 1995]

Chromosome 9p Allelic Loss and *p16/CDKN2* in Breast Cancer and Evidence of *p16* Inactivation in Immortal Breast Epithelial Cells[1]

Andrew J. Brenner and C. Marcelo Aldaz[2]

The University of Texas M. D. Anderson Cancer Center, Department of Carcinogenesis, Smithville, Texas 78957

ABSTRACT

To define the extent of involvement of chromosome 9p in breast carcinogenesis, we performed microsatellite length polymorphism analysis of markers spanning this region. Of 24 primary breast carcinomas analyzed, we observed a high frequency (58%) of loss of heterozygosity or allelic imbalance affecting subregion 9p21–22. Mutational analysis of *CDKN2* (*p16*) was performed to determine whether this gene was the target of such alterations. Of 21 tumors analyzed, only 1 showed a mutation of probable consequence, suggesting that *CDKN2* appears not to be the target of loss of heterozygosity and indicating the possible existence of another tumor suppressor gene within this region. Additionally, since it has been suggested that some *CDKN2* deletions and mutations could be due to an *in vitro* phenomenon, four immortal breast cell lines derived from normal epithelium, MCF10F, MCF12F, 184A1, and 184B5, were examined for loss or mutation of *CDKN2*. Two lines (MCF10F and MCF12F) showed homozygous deletions of *CDKN2*, and one (184A1) revealed a hemizygous deletion and a nonsense mutation in the remaining allele. This could imply an important role of *CDKN2* in the control of immortalization or *in vitro* adaptation and is the first evidence of such in nontumor-derived cell lines. Additionally, this is the first report of frequent loss of heterozygosity in the 9p21–22 chromosome subregion of uncultured primary breast tumors.

INTRODUCTION

Breast cancer is the most common malignancy in American women, affecting as many as one in eight, and responsible for as many as one in five cancer-related deaths of women (1, 2). It has, therefore, become essential to define the molecular events resulting in breast carcinogenesis. Various chromosomes have been observed to be affected by a higher frequency of structural or numerical abnormalities in breast cancer. At the molecular level, several somatic mutations have also been described affecting various oncogenes and tumor suppressor genes. Allelic losses at variable frequencies have been reported for numerous chromosome subregions, the most common being 1p34–36, 1q23–32, 3p21–25, 6q, 7q31, 11p15, 13q14, 16q, 17p13, 17q, and 18q (3–14). LOH[3] has been classically viewed as the indirect evidence for the possible existence of a tumor suppressor gene within a region affected by loss of alleles.

One chromosome region that undergoes hemizygous and homozygous deletions in a variety of tumor types is 9p21–22 (15–18). Detailed analysis of this region has shown that it contains an inhibitor of the cell cycle, the cyclin dependent kinase-4 inhibitor (*CDKN2*) gene, commonly referred to as *p16* (19). Further analysis has revealed that this gene is frequently homozygously lost or deleted in cell lines derived from many tumor types, including astrocytoma (82%), bladder carcinoma (33%), lung carcinoma (25–32%), glioma (71–88%), melanoma (58–62%), renal carcinoma (56%), and breast carcinoma (60%; Refs. 19–21). This evidence implicated *CDKN2* as a putative tumor suppressor. However, since these studies were performed with cells grown *in vitro* and since losses within 9p are infrequent in some tumor types, the relevance of *CDKN2* in these tumors was still questionable. To address this question, mutational analysis of *CDKN2* by sequencing has been conducted revealing alterations in uncultured tumors including esophageal carcinoma (52%), lung carcinoma (30%), and pancreatic carcinoma (38%), thus supporting the role of *CDKN2* as a tumor suppressor (22–24). Conversely, some tumor types have shown none or few mutations, while lines derived from these types have shown high frequency of *CDKN2* deletions (25).

To ascertain whether chromosomal region 9p21–22 is also affected in breast cancer and further determine if *CDKN2* plays a role in breast carcinogenesis, we performed allelotyping of the short arm of chromosome 9, SSCP analysis of *CDKN2* exon 1, and sequencing of *CDKN2* exon 2 in 21 uncultured primary breast carcinomas. Additionally, since it has been suggested that some *CDKN2* deletions and mutations are due to an immortalization or *in vitro* adaptation phenomenon, we analyzed four immortal breast epithelial lines derived from normal epithelium for loss or mutation of *CDKN2*.

MATERIALS AND METHODS

Tissue Samples, Cell Lines, and DNA Extraction. Normal and tumor breast samples were obtained from the Cooperative Human Tissue Network. Samples were snap frozen with liquid nitrogen less than 1 h after surgery. Cell lines MCF-10F (CRL10318), MCF-12F (CRL10783), 184A1 (CRL8798), and 184B5 (CRL8799) were purchased from American Type Culture Collection (Rockville, MD) and cultured as described elsewhere (26, 27). Total genomic DNA was isolated using phenol:chloroform:isoamyl (25:24:1) in Phase Lock Gel tubes (5 Prime→3 Prime, Boulder, CO), according to a standard protocol (28), and precipitated with 2.5 volumes of ethanol.

Microsatellite Length Polymorphism and *CDKN2* Deletion Analysis. PCR was performed using 60 μM individual end-labeled primer sets, 175 μM corresponding cold primer, 100–300 ng of template, 2 mM $MgCl_2$, 300 μM deoxynucleotide triphosphates, 1 × *Taq* buffer (Promega), 10% DMSO, and 1.5 units of *Taq* polymerase (Promega). Amplification was done using a hot-start protocol; DNA, primers, DMSO, and H_2O were heated to 95°C for 5 min and then brought to 80°C. Remaining ingredients were added, and cycling then proceeded with no extension step. Primers used were: *D9S199, D9S157, D9S171, D9S169, D9S165,* or *D9S15* (Research Genetics, Huntsville, AL). Products were resolved on 7% sequencing gel, and autoradiograms were developed after 12–48 h exposure. Partial LOH and allelic imbalance were considered significant only if the signal intensity of one allele was diminished by approximately one-half or more of its normal intensity in relation to the remaining allele; complete LOH was defined as a decrease of more than 90% in the signal intensity of one allele relative to the other.

Loss of *CDKN2* was determined by duplex PCR using unlabeled primers for exon 2 mentioned below for sequencing, simultaneously with control microsatellite marker *D13S155*. Amplification was done as described above for microsatellites. The resulting products were resolved by electrophoresis on a 2–3% NuSieve (FMC) agarose gel in 1X TAE.

Sequencing and PCR-SSCP. Exon 1 was analyzed by PCR-SSCP and sequenced as reported previously (29). Exon 2 of the *CDKN2* gene was amplified by PCR using the primers 5′-ACCATTCTGTTCTCTCTGGC-3′ and 5′-CTCAGATCATCAGTCCTCAC-3′. Amplification products were resolved on 2% NuSieve agarose (FMC), bands were excised, and DNA was recovered using a PCR Preps kit (Promega). Sequencing reactions were then

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[2] To whom requests for reprints should be addressed.

[3] The abbreviations used are: LOH, loss of heterozygosity; SSCP, single-strand conformational polymorphism; CDK4, cyclin-dependent kinase-4.

performed using a fmol cycle sequencing kit (Promega) with the same primers and inside primers 5′-CACCAGCGTGTCCAGGAA-3′ and 5′-CGATGCCT-GGGGCCGTCT-3′. Because of the GC-rich nature of the sequence, excess deoxynucleotide triphosphates and 1 unit Terminal Transferase (USB) were added, and nonterminated products were extended at 37°C for 30 min. Products were resolved on a 5% sequencing gel and autoradiograph developed after 12–96 h exposure.

RESULTS AND DISCUSSION

Previous allelotypic studies have not implicated the short arm of chromosome 9 as a frequent target for allelic losses in breast cancer (3–14). But most of these earlier studies were based on RFLP analysis by means of Southern blotting with a very limited number of molecular markers for chromosome 9p (6, 14). Interestingly, more recent studies using microsatellite length polymorphism analysis have demonstrated 9p loss in a variety of tumor types. These include gliomas (30), melanomas (31), bladder carcinomas (32), lung carcinomas (17), and most recently, preinvasive and malignant head and neck squamous cell carcinomas (15). These losses have been localized to region 9p21–22 (33). To ascertain whether this same region is affected in breast cancer, we used five polymorphic microsatellite markers spanning the p arm and one marker on the q arm to assess LOH at chromosomal region 9p21–22. Marker *D9S199* is located in the telomeric region of 9p; 10 cM proximal in 9p21–22 is marker *D9S157*; another 7cM proximal in 9p21 is marker *D9S171*; 7 cM further is *D9S169*, and *D9S165* is located between 9p13 and marker *D9S15* at 9q13 (Fig. 1). Interestingly, of the 24 tumors we analyzed, allelic imbalances or LOH was observed in 14 tumors (58%; Fig. 1), and at least one-half of the tumors showed allelic imbalance or LOH of multiple markers (Figs. 1 and 2). Most losses observed affected markers *D9S169* and *D9S171*, which showed frequencies of LOH or imbalance of 58 and 53% of informative cases, respectively. Although a few cases clearly involve the entire short arm (A12, A19, and A23) or chromosome (A11), the majority of cases contain losses that begin or end on either side of these two markers. This indicates that the minimum area of overlap includes these two markers and that the target area lies somewhere in between. To our knowledge, there have been no previous reports of LOH or other alterations on chromosome 9p in uncultured primary breast tumors. However, our analysis indicates a high incidence of allelic abnormalities at chromosomal region 9p21 in breast cancer.

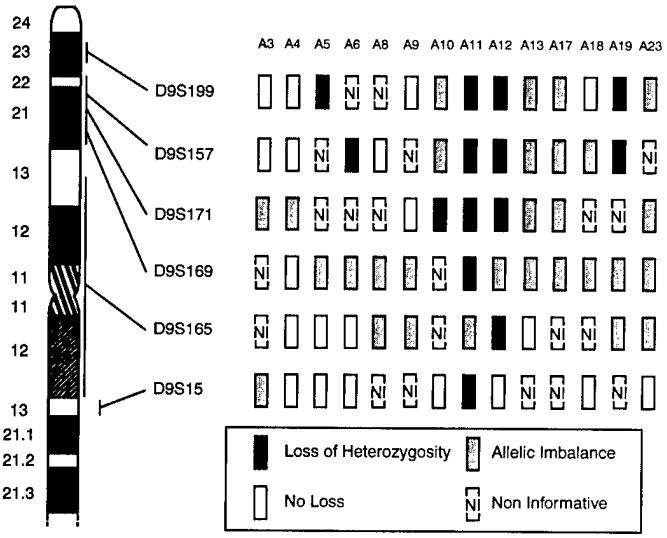

Fig. 1. Chromosome 9p allelic losses and imbalances in uncultured breast carcinomas.

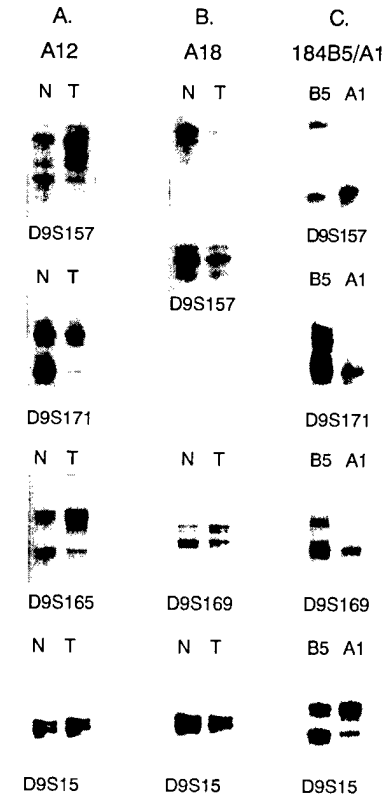

Fig. 2. Representative microsatellite analysis of two primary breast carcinomas (A12 and A18) and two chemically immortalized breast epithelial cell lines (184A1 and 184B5). *A*, tumor A12 shows loss of heterozygosity of three markers on chromosome arm 9p (*D9S157, D9S171,* and *D9S165*) while retaining heterozygosity at 9q13 (*D9S15*). *B*, tumor A18 shows an allelic imbalance of two 9p markers (*D9S157* and *D9S169*) and retains heterozygosity at 9q13 (*D9S15*). *C*, chemically immortalized cell line 184A1 shows complete loss of heterozygosity at three 9p markers as well as an allelic imbalance at 9q13 (*D9S15*).

On the basis of this relatively high rate of alterations at subregion 9p21 and on the basis of the previous report of Kamb *et al.* (19) indicating *CDKN2* as one obvious target of allelic loss in this region, we decided to search for alterations in the *CDKN2* gene. All tumors were examined, except tumors A22 and A24 (which incidentally showed no alteration of chromosome 9p) and tumor A11, to which sufficient DNA was not available. By direct sequencing of exon 2, we found only three alterations, of which only one was of probable consequence. This was a missense mutation observed in codon 75 (tumor A1; Fig. 3*A*), resulting in an amino acid change from histidine to tyrosine. This mutation had been reported previously (29) and is not suspected of being a polymorphism. Of additional interest is that this tumor (A1) showed no allelic loss on the p arm of chromosome 9 (Fig. 1). Of the two other mutations found, one was a missense change from alanine to threonine in codon 140 (tumor A9), a frequently reported probable polymorphism apparently not involved in cancer (34), and the second, a silent mutation in codon 65, resulting in no change of amino acid sequence (tumor A13). The remaining 18 tumors revealed no mutations by sequencing of exon 2. Although previous reports indicate that the majority (~90%) of mutations of *CDKN2* occur in exon 2 (19, 34), we performed PCR-SSCP analysis of exon 1 to be sure no mutations were present. To facilitate detection, two different conditions were used as described in a previous report (29). Nevertheless, we observed no mobility shifts in any of the 21 tumors analyzed (data not shown). To corroborate our PCR-SSCP results, we also performed sequencing of exon 1 in some tumors (A10, A12, A16, and A23) and found no base changes.

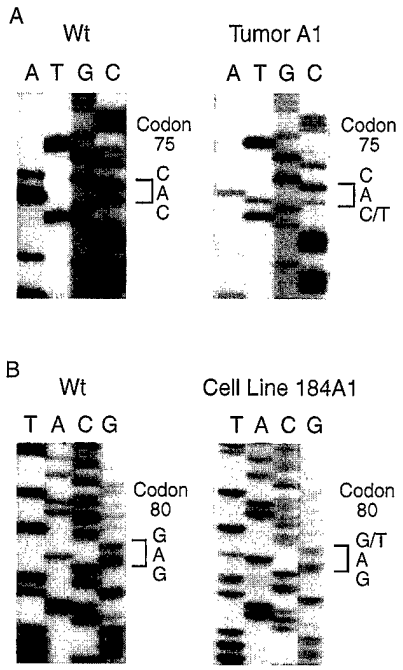

Fig. 3. Mutational analysis by sequencing of *CDKN2* exon 2. *A*, tumor A1 shows a missense mutation at codon 75, resulting in an amino acid change from histidine to tyrosine. *B*, immortal nontumorigenic breast epithelial cell line 184A1 shows a nonsense mutation at codon 80, resulting in a truncated transcript.

Despite the high rate of allelic imbalance affecting the 9p21 region, the aforementioned results indicate that *CDKN2* is not frequently mutated in these breast tumors. This low rate of mutation is in agreement with a previous report that used PCR-SSCP exclusively (35) and suggests that mutations of *CDKN2* are not a critical event in breast carcinogenesis and that this gene is probably not the target of allelic loss.

A discrepancy exists between the high rate of loss of *CDKN2* in cell lines derived from certain tumor types and the rarity of alterations in the corresponding primary tumors themselves. This is exemplified by the contradiction between the finding of Kamb *et al.* (19) of *CDKN2* loss in >60% of cell lines derived from breast carcinomas and our findings of few mutations or homozygous loss in primary breast carcinomas themselves. To help address this issue, we analyzed four nontumorigenic immortal cell lines. Two of these cell lines, MCF-10F and MCF-12F, are spontaneously immortalized cell lines produced by long-term culture of normal mammary epithelial tissue (26). The other two cell lines, 184A1 and 184B5, are independently derived from normal mammary epithelial cells from one patient and immortalized by treatment with benzo(*a*)pyrene (27). Of the four cell lines, both MCF-10F and MCF-12F showed complete loss of both *CDKN2* alleles. As can be observed in Fig. 4, both lines failed to amplify exon 2 of *CDKN2* in the presence of a control primer set in a duplex PCR reaction. Furthermore, in independent testing using radioactively labeled primers for PCR-SSCP analysis, both lines failed to amplify exon 1 of *CDKN2* (data not shown). Additionally, both cell lines were also analyzed for the presence of microsatellite markers spanning chromosome 9p. We observed that markers proximal and distal to *CDKN2* retained heterozygosity (data not shown). However, marker *D9S171* (heterozygosity score, 0.79) was found to be noninformative in both cell lines. Since other normal tissues from these patients were not available, we could not determine the constitutive genotype at this locus. Nevertheless, the homozygous loss of *CDKN2* in MCF10F and MCF12F appears to be the consequence of localized deletion events affecting both homologous chromosome arms. Interestingly, karyotypic analysis has shown (26) that cell line MCF-10F has a balanced reciprocal translocation, t(3;9)(3p13:9p22). However, it is not known whether this translocation "correlated in time with the acquisition of immortality," although it is known to have happened early in culture (26).

Further evidence suggesting that *CDKN2* may be a specific target for abnormalities while *in vitro* was provided by direct sequencing mutational analysis of the chemically [benzo(*a*)pyrene] immortalized line 184A1, which revealed a nonsense mutation at codon 80 (Fig. 3*B*). In addition, LOH at all three microsatellite markers in the 9p21 subregion was observed (Fig. 2*C*). These findings appear to indicate that one allele of *CDKN2* in 184A1 was deleted and that the remaining allele was inactivated through a nonsense mutation. Furthermore, it is possible that the mutation observed is the direct result of the benzo(*a*)pyrene treatment. We did not observe, however, any mutations of exons 1 or 2 of *CDKN2* in the 184B5 cell line by direct sequencing analysis. The possibility does exists that a mutation or deletion exists in another gene, such as the retinoblastoma gene, thereby circumventing the need for *CDKN2* inactivation. Experiments are in progress to address this point.

Previous work has shown that *CDKN2* alterations are at least three times more common in tumor-derived cell lines than in uncultured tumors. However, to our knowledge, there has been no analysis of *CDKN2* in cell lines derived from normal tissue. Here we show that *CDKN2* was affected in three of four cell lines (75%) derived from "normal tissue." This may help explain the aforementioned discrepancy between the high rate of loss of *CDKN2* in cell lines derived from certain tumor types and the rarity of alterations in the corresponding primary tumors themselves. It would be reasonable to assume that to escape senescence, cell lines would need to mutate or lose genes that play an important role in restricting cell cycle progression. *CDKN2* has been shown to be an important inhibitor of the cell cycle that acts to block progression through G_1 by inhibiting the kinase activity of CDK4. The kinase activity of CDK4 is able to phosphorylate important regulatory proteins, such as Rb, and prevents them from binding and inactivating their associated transcription factors, thereby stimulating growth. Should CDKN2 be deleted or inactivated by mutation, *CDK4* would be free to propel the cell through the cell cycle and into cell division. This would be analogous to the loss of p53 in immortalization (36), since p53 acts to regulate cell cycle progression through transcriptional activation of $p21^{waf-1}$, an inhibitor of CDK2. Thus, the implication is that although *CDKN2* may not play a significant role in breast carcinogenesis, it may be important in the control of immortalization or *in vitro* adaptation. This explanation is supported by recent findings of Loughran *et al.* (37) in

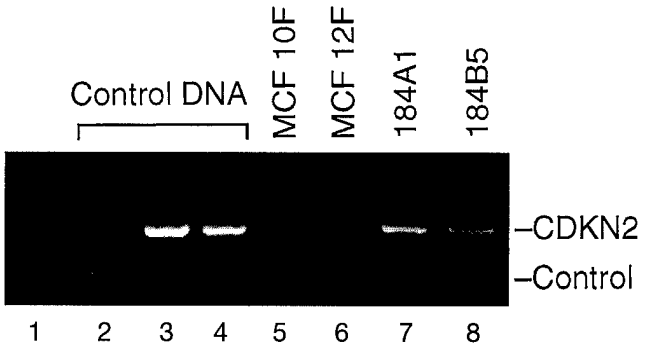

Fig. 4. Duplex PCR analysis of *CDKN2* showing homozygous deletion of exon 2 in nontumorigenic immortal breast epithelial lines MCF10F and MCF12F. *Lane 1*, molecular weight markers; *Lane 2*, control DNA with primer *D13S155*; *Lane 3*, control DNA with primer for *CDKN2*; *Lane 4*, control DNA with duplex PCR (*D13S155* and *CDKN2*); *Lanes 5–8*, duplex PCR on cell lines as indicated.

which loss of the 9p21 subregion was found to correlate with the acquisition of an immortal phenotype of neoplastic human head and neck keratinocyte cell lines. Most of the immortal lines analyzed in that report were derived from advanced tumors. Although loss of *CDKN2* does not constitute evidence that this is a senescence gene, our finding of loss in two nonneoplastic lines and loss with mutation of the remaining allele in another adds weight to this prospect.

In summary, in the present study, the frequency of alterations of the 9p21 region in uncultured breast tumors was high (≈60%), while the rate of mutation of *CDKN2*, a tumor suppressor gene located in this region, was significantly low to suggest that it is not the target of alteration in breast cancer. However, the possibility exists that the frequency of alterations of *p16/CDKN2* is actually higher since some mutations are located outside of the coding region, or hypermethylation of this gene could affect expression. Alternatively, detection of mutations might be masked by DNA from other cells in the heterogeneous tumor cell population or by infiltrating normal tissue, as suggested by Kamb *et al.* (38). However, studies using methodology similar to ours in other uncultured tumor types have been conducted and have revealed a significant number of mutations (22–24). Therefore, we can assume that most mutations are not escaping detection. Alternatively, it may be that another tumor suppressor gene of significance in breast cancer could reside within this chromosomal subregion. This is supported by findings of similar discrepancies in primary tumors of other tissue types (39). Further studies are needed to better define the extent of involvement of *CDKN2* in tumorigenesis. Studies are under way in our laboratory to specifically determine if loss of *CDKN2* is sufficient for immortalization and whether restoration of *CDKN2* expression in the aforementioned lines will induce senescence.

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Preferential Loss of Expression of *p16^{INK4a}* Rather Than *p19^{ARF}* in Breast Cancer[1]

Andrew J. Brenner, Abhaya Paladugu, Hui Wang, Olufunmilayo I. Olopade, Martin H. Dreyling, and C. Marcelo Aldaz[2]

Department of Carcinogenesis, The University of Texas M. D. Anderson Cancer Center, Research Division, Smithville, Texas 78957 [A. J. B., A. P., H. W., C. M. A.], and University of Chicago Medical Center, Chicago, Illinois 60637 [O. I. O., M. H. D.]

ABSTRACT

The tumor suppressor *p16^{INK4a}* has been shown to be inactivated in numerous cancer lines and primary tumors. Recently, we reported loss of heterozygosity of the region in which *p16^{INK4a}* is located in more than one-half of primary breast tumors. However, mutational analysis of these same tumors revealed mutation of *p16^{INK4a}* to be infrequent. Other possible modes of inactivation, such as *de novo* methylation and homozygous deletion, have since been shown to occur in numerous neoplasias. Furthering the complexity of this locus, a transcript overlapping the *p16^{INK4a}* coding sequence and encoding a novel peptide with growth-suppressive activity, *p19^{ARF}*, has been described. To clearly elucidate the target of aberrations affecting this subchromosomal region and approximate frequency in breast cancer, we performed a comprehensive study including *p16* deletion analysis by means of interphase chromosomal fluorescence *in situ* hybridization, methylation analysis of the first exon encoding *p16^{INK4a}* (exon 1α), mutational analysis of exon 1β by single-strand conformational polymorphism analysis of *p19^{ARF}* transcripts, and expression of both α and β transcripts by reverse transcription PCR. Homozygous deletion of *p16*, as determined by interphase chromosomal fluorescence *in situ* hybridization, was observed in 3 of 18 (17%) tumors analyzed, whereas *de novo* methylation of exon 1α was observed in an additional 17% (4 of 23). Reduced expression of *p16^{INK4a}* was observed in 11 tumors (48%), including all those in which homozygous deletion or complete methylation was observed. No mutations of exon 1β were detected, and expression of its transcript was variable, with 13% demonstrating decreased expression and 17% demonstrating overexpression. These results further support *p16^{INK4a}* as a target of inactivation in the 9p21 region for breast cancer.

INTRODUCTION

Chromosomal subregion 9p21 has been shown to undergo hemizygous and homozygous deletion in a variety of tumor types (1–3). Recently, we have reported hemizygous loss to occur frequently in breast cancer (58%) as well (4). Previous analysis of this region had shown it to contain *p16^{INK4a}*, an inhibitor of cyclin-dependent kinases, commonly referred to as *p16* (5, 6). Nevertheless, when we subjected these same tumors to mutational analysis of *p16*, we observed few tumors with sequence alterations of consequence, thus suggesting that *p16* may not be the target of such anomalies in breast cancer (4). However, recent reports have indicated that mutation may not be the primary mechanism of inactivation of the *p16* gene in many tumor types (7–12).

Recently an alternative transcript encoded from the same second and third exons encoding *p16* and using the same splice site, but with a separate promoter and alternative first exon, now referred to as exon 1β, has been described (13, 14). This alternative transcript is abundant in various tissue types (13, 14) and is translated in the mouse from an alternative reading frame, resulting in a protein of 19 kilodaltons with cell cycle-arresting capacity, now termed p19^{ARF} (15). Thus, the *p16* locus appears to be complex, with two overlapping transcripts translated from distinct reading frames, resulting in two polypeptides, p16^{INK4a} and p19^{ARF}, each able to induce cell cycle arrest. Because the transcripts of these two polypeptides partially overlap, it may be that alterations that affect one may also affect the other. Therefore, the possibility exists of p19^{ARF} being a protein with tumor-suppressive function being targeted for inactivation as well. A comprehensive analysis of the aberrations affecting the *p16* and *p19* genes and expression of transcripts *in vivo* should help clarify these issues.

To help elucidate the role of aberrations affecting these two genes in breast cancer, we have performed a comprehensive analysis of inactivation and expression in a series of primary breast carcinomas. To that end, we performed IC-FISH[3] deletion analysis of the *p16* region, methylation analysis of the 5′ region of the primary first exon, SSCP analysis of the alternative exon 1β transcript, and expression of both transcripts (α and β) by semiquantitative RT-PCR. Taken together with previously determined microsatellite polymorphism LOH analysis of 9p21 and *p16* mutational analysis in these same tumors (4), we believe we have obtained a more complete account of p16^{INK4a} and putative p19^{ARF} involvement in breast tumorigenesis.

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[2] To whom requests for reprints should be addressed, at Department of Carcinogenesis, The University of Texas M. D. Anderson Cancer Center, Science Park, Research Division, Smithville, TX 78957. Phone: (512) 237-2403; Fax: (512) 237-2475.

[3] The abbreviations used are: IC-FISH, interphase chromosomal fluorescence *in situ* hybridization; SSCP, single-strand conformational polymorphism; RT, reverse transcription; LOH, loss of heterozygosity; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

MATERIALS AND METHODS

Tissue Samples, DNA Extraction, and RNA Extraction. Normal and tumor breast samples were obtained from the Cooperative Human Tissue Network. All samples were invasive ductal carcinomas with the exception of cases 24 (ductal carcinoma *in situ*) and 32 (mucinous adenocarcinoma). Samples were snap frozen with liquid nitrogen less than 1 h after surgery. Regions dense in tumor cells were identified by visual inspection and comparison with H&E slides as necessary. Total genomic DNA was isolated using phenol:chloroform:isoamyl (25:24:1) in Phase Lock gel tubes (5 Prime → 3 Prime, Inc., Boulder, CO) according to standard protocol and precipitated with 2.5 volume ethanol. Total RNA was isolated using an RNEasy Total RNA kit (Qiagen, Chatsworth, CA) as per the manufacturer's instruction.

IC-FISH. Probe pHUR98, a variant satellite 3 sequence that specifically hybridizes to the heterochromatic region of chromosome 9, was used to assess chromosome 9 copy number. p16cos is a contig of eight cosmids encompassing a 250-kb region around *p16*, obtained by screening a flow-sorted chromosome 9 library (10) and used to determine *p16* copy number. Probes were amplified and labeled by sequence-independent amplification (16) and either biotin-11-dUTP (Enzo Diagnostics) labeled (centromeric probe pHUR98) or digoxigenin-11-dUTP (Boehringer Mannheim) labeled (p16cos contig). Dual-color chromosomal FISH was performed as described previously (10). Biotinylated probes were detected with Texas red-avidin (Vector Laboratories), and digoxigenin-labeled probes were detected with FITC-conjugated antibodies (Boehringer Manheim). Gray-scale images corresponding to each fluorochrome were captured from tumor cell interphases selected at random, using a Photometrics (Tucson, AZ) CCD cooled camera. Pseudocolor composite images were analyzed using Oncor (Gaithersburg, MD) Image software. A total of 150 intact tumor cells was analyzed by three independent observers (A. B., A. P., and C. M. A.). Cells with no visible p16cos signal were interpreted as nullizygous for p16 and labeled as total deletions, whereas cells displaying a single copy of the p16 region as well as cells with relative deletion (fewer copies of the p16 region than centromeric pHUR89) were labeled as partial deletions. To avoid overinterpretation of incomplete hybridization, based on analysis of normal human lymph nodes, tumor cell populations were not included in scoring of deletions unless present at $\geq 10\%$.

Southern Hybridization. Methylation analysis was performed as described previously (7, 17). Briefly, 10 μg of genomic DNA were digested with a flanking site enzyme (either *Eco*RI or *Hin*dIII) and a methylation-sensitive endonuclease (*Sac*II, *Sma*I, or *Eag*I), ethanol precipitated, resuspended, and resolved in a 1% agarose gel overnight. DNA was transferred to a Zeta-Probe nylon membrane (Bio-Rad, Richmond, CA) and hybridized with a 340- or 280-bp [α-^{32}P]dCTP random prime-labeled PCR fragment including exon 1α, as described (7, 17). Autoradiographs were obtained following 2–4 days of exposure.

RT-PCR. Five μg of total RNA were used for first-strand cDNA synthesis with Superscript II reverse transcriptase (Life Technologies, Inc., Gaithersburg, MD) as per the manufacturer's instructions. Following RT, each sample was subjected to analysis of GAPDH levels as control of mRNA quantity by PCR amplification using a GAPDH Positive control primer set (Stratagene, La Jolla, CA). RT-PCR of both *p16* α and β transcripts was performed as described previously (13). An initial experiment was performed to validate the quantitative nature of the RT-PCR, as reported previously (13), and was found to concur (data not shown). Mean normal expression was obtained by analysis of four normal reduction mammoplasty breast samples. Signal intensities in all cases were analyzed and quantified with a Molecular Dynamics PhosphorImager. A relative value of 1.0 was assigned to the ratios of α:GAPDH and

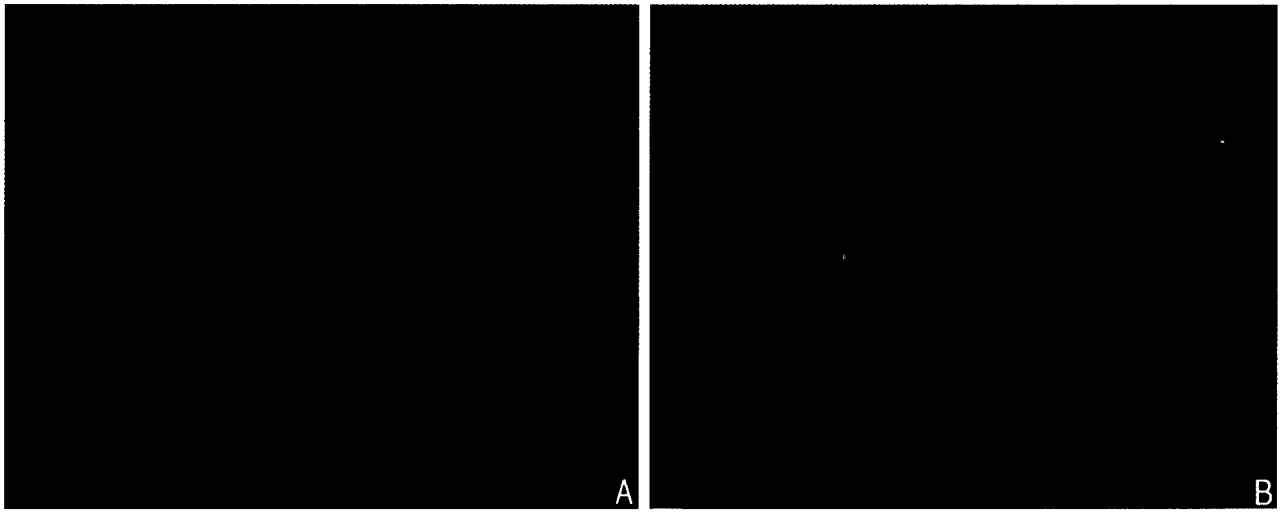

Fig. 1 Bi-color chromosome FISH performed with chromosome 9 centromere-specific probe (*red*) and *p16*-specific probe (*green*). *A*, representative interphase nuclei from a normal human lymph node displaying two alleles of both the centromeric region of chromosome 9 (*red*) and the p16cos region (*green*). *B*, representative interphase nuclei from human breast carcinoma sample 28. Note the heterogeneity of the tumor with cells showing no p16cos (*green*) signal, one p16cos signal, or two p16cos signals.

β:GAPDH based on normal mean, and subsequent samples were normalized accordingly. All experiments were performed in triplicate, and SDs were calculated.

SSCP. Amplification of β transcripts was performed as described above. RT-PCR products were diluted 2:1 in denaturing loading buffer (95% formamide, 10 mM NaOH, and 0.1% xylene cyanol) and resolved in 6% polyacrylamide using two conditions: 10% glycerol and 1× Tris-borate EDTA at 16 W for 16 h; and 5% glycerol and 0.5× Tris-borate EDTA at 8 W for 8 h.

RESULTS AND DISCUSSION

***p16^INK4a* Locus Copy Number.** The 9p21 chromosomal subregion has been shown to undergo LOH in a variety of neoplasias (1, 18, 19). Although the *p16^INK4a* gene is known to be located within this region, mutational analysis of the *p16^INK4a* gene in breast tumors revealed infrequent mutations (<5%; ref 4). To determine whether these tumors may have alternatively incurred homozygous deletion of the *p16* region, and to what extent, we performed dual-color IC-FISH using the 250-kb p16cos contig and a centromeric probe for chromosome 9 copy number (Fig. 1). We observed total or partial deletion of the *p16* chromosomal region in 61% (11 of 18) of breast tumors. This result is comparable to our aforementioned analysis of the same tumor set by PCR-based microsatellite length polymorphism analysis (4). Specifically, three cases (17% of the total) displayed tumor cell subpopulations with total deletion of the p16cos region. Subsequent analysis by RT-PCR showed low to absent expression in both of the two cases for which sufficient material was available. However, although partial deletion was more common (11 cases) than total deletion, subsequent expression analysis did not show a strong association between partial deletion and loss of expression (Table 1). Tumor cell populations displaying hyperdiploidy of chromosome 9 were observed in eight (45%) of the tumors analyzed. No tumors showed evidence of significant chromosome 9 monosomy.

To our knowledge, this is the first report of homozygous deletion of the *p16* region in primary breast carcinomas through the use of *in situ* hybridization. Although two previous studies have been conducted on breast carcinomas by Southern analysis, cumulatively no homozygous deletions were reported of the 21 breast tumors analyzed (9, 20). Because breast cancer samples may also contain a significant portion of normal, nonneoplastic stromal or epithelial cells or heterogeneous tumor cell populations, it may be that Southern analysis is not of sufficient sensitivity for determinations of homozygous loss. However, another study based on microsatellite analysis reported homozygous loss at a frequency comparable to that reported here (21).

Hypermethylation of *p16^INK4a* Exon 1α. Exon 1 of the *p16^INK4a* gene contains a documented CpG island, which has been shown to be unmethylated in normal tissue and hypermethylated in certain tumor types at varying incidence (7–9). To establish the methylation status of the 5′ region, total genomic DNA was digested with a combination of a flanking site endonuclease and a methylation sensitive endonuclease, as described previously (9). Twenty-three tumors were analyzed, of which patterns consistent with partial or total methylation were observed in four (17%; Table 1 and Fig. 2). Two tumors (tumors 30 and 41) showed methylation with multiple restriction enzymes (*Sac*II and *Sma*I), whereas two others (tumors 16 and 44) revealed methylation with only one enzyme (*Eag*I and *Sac*II, respectively). Of those tumors showing methylation, three (tumors 16, 30, and 41) displayed patterns consistent with methylation of all possible endonuclease sites in that region, whereas one (tumor 44) displayed methylation of a single site. The remaining 19 tumors revealed no pattern consistent with hypermethylation. These results are consistent with a previous report of methylation in primary tumors of the breast, although the frequency observed here (17%) is somewhat lower than the frequency reported previously (31%) by Herman *et al.* (9).

Table 1 Analysis of *p16^INK4a* and *p19^ARF* in breast cancer

Tumor no.	Deletion status (% of cells)[a] Total	Partial	Methylation[b]	α (p16) expression[c]	β (p19) expression[c]
6	68	11	−	↓↓	↓
28[d]	44	15	−	↓↓	N
48[d]	10	50	−	nd	nd
16	nd	nd	+	↓↓	↓↓
30[d]			+	↓	N
41[d]	nd	nd	+	↓↓	↑
14		11	−	↓↓	N
18			−	↓↓	N
4	nd	nd	−	↓↓	N
8		44	−	↓	N
10			−	↓	N
22[d]		47	−	↓	N
26[d]	nd	nd	−	N	↓
44[d]	nd	nd	+/−	N	N
11[d]		36	nd	N	N
24[d]			−	N	N
32[d]			−	N	N
34[d]			−	N	N
36[d]			−	N	N
38[d]	nd	nd	−	N	N
50[d]		23	−	N	N
20[e]		28	−	N	↑
2		23	−	↑	↑
54		23	−	↑	↑

[a] Determined by the relative signal of p16cos to chromosome 9 centromeric probe in IC-FISH analysis. Tumor cell populations were not included in scoring of deletions unless present at ≥10% (see "Materials and Methods"). nd, not determined.

[b] Methylation status determined by Southern analysis as described in "Materials and Methods." −, no methylation; +, methylation; +/− partial methylation.

[c] Expression determined by RT-PCR analysis as described in "Materials and Methods." ↓↓, <10% of normal mean expression; ↓, <30% of normal mean expression; ↑, >300% of normal mean expression; N, normal expression.

[d] Wild-type p16 exon 2 (4).

[e] Polymorphism base 140, exon 2 (4).

Expression of *p16^INK4a* α and β Transcripts. As previously suggested by Stone *et al.* (13), the similarity in size and sequence of the α and β transcripts may have complicated previous efforts to measure *p16* RNA levels by Northern blot (13) in different neoplasias. Only an analysis of transcripts using the unique sequences of exon 1α would be able to asses the true levels of *p16* expression. Additionally, because inactivating events that target *p16* may also affect the alternative β transcript, and because we know the alternative β transcript to

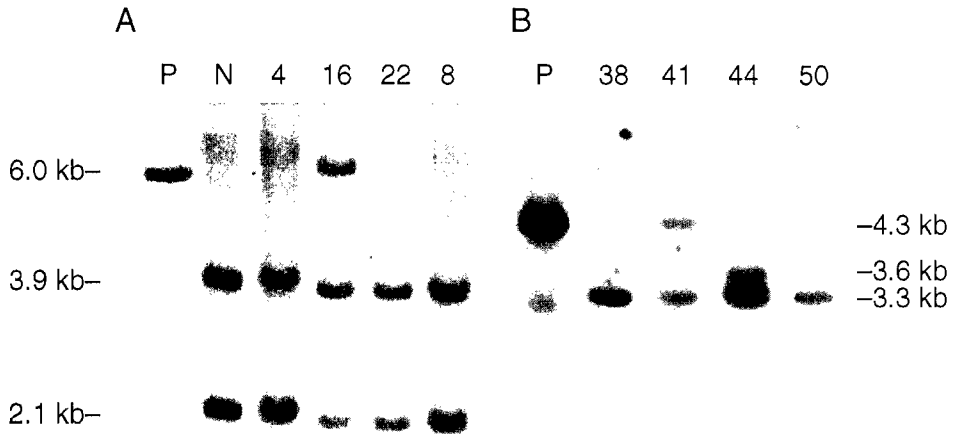

Fig. 2 Representative Southern analysis of *p16* exon 1α in primary breast carcinomas with methylation-sensitive restriction enzymes. *A*, analysis of methylated line T47D (*P*, positive control; Ref. 9), normal breast DNA (*N*), and four breast carcinomas with *Hin*dIII and *Eag*I reveal methylation of sample 16. *B*, analysis of methylated line T47D (*P*) and four breast carcinomas with endonucleases *Eco*RI and *Sac*II reveal complete methylation of tumor 41 and partial methylation of tumor 44.

encode $p19^{ARF}$ and to have growth-suppressive effects in murine cells *in vitro*, it would be advantageous to evaluate both *p16* and alternative β transcript expression independent of one another.

Expression levels of *p16* α and β transcripts in 23 tumors were determined by RT-PCR analysis. Expression levels of each transcript were subsequently compared with mean normal expression of a panel of four normal breast samples. Expression of both the *p16* primary α and alternative β transcripts in breast tumors was varied (Table 1 and Fig. 3, *A* and *B*). Six of 23 (26%) showed expression of $p16^{INK4a}$ at levels less than 10% of the normal mean, whereas another 5 (22%) revealed levels of expression from 10 to 30% of the normal mean (*i.e.*, greater than 70% reduction in normal expression; see Fig. 3). Loss of expression in many of these tumors can be accounted for by either hypermethylation (tumors 16, 30, and 41) or homozygous deletion (tumors 6 and 28). However, inactivation by either of these mechanisms was not observed in some cases in which loss of expression was observed, indicating that other modes of inactivation could be operative. Moreover, a previous report of $p16^{INK4a}$ expression by immunohistochemistry suggested loss of expression in as many as 65% of breast tumors (22), indicating that inactivating events might be possible at a posttranscriptional stage as well. We also observed that two additional carcinomas, tumors 2 and 54, displayed what appears to be overexpression of $p16^{INK4a}$, concomitant with β overexpression. Previous analyses in numerous lines have indicated that the overexpression of *p16* can be associated with retinoblastoma protein inactivation (23). However, no precedent of this association has been described *in vivo*. Nonetheless, such overexpression may be deemed aberrant.

Analysis of β transcripts showed great variability in expression, with apparent overexpression to be as prevalent as lack of expression. One of the 23 tumors analyzed revealed undetectable levels of expression (tumor 16). Two additional carcinomas (tumors 6 and 26) showed expression at less than 30% of the normal mean. Incidentally, the two cases in which the lowest expression of β was observed (tumors 6 and 16), both displayed α loss, and by distinct mechanisms. Loss of expression in tumor 6 appeared to be through homozygous deletion, whereas loss of expression in tumor 16 appeared to be through methylation and LOH (4). However, methylation of exon 1α only explains loss of expression of the primary α transcript. Perhaps in some cases, such as tumor 16, the methylation of the 5′ region of exon 1α is indicative of the hypermethylation of the entire locus, and as such, the 5′ region of exon 1β also could be hypermethylated. Because the 5′ region of exon 1β from −180 to +266 bp contains 70% GC content and a CG:GC ratio of 0.71, thus defining a CpG island, this probability exists. Of additional interest, four tumors (tumors 2, 20, 41, and 54) showed considerably high levels of β ($p19^{ARF}$) expression between 3- and 5-fold greater than the normal mean. What level of increased expression can be considered significant and the possible implications of such overexpression have yet to be determined.

The possibility of $p19^{ARF}$ being tumor suppressive in function has been postulated previously (13, 15). However, this has not yet been shown, and an analysis of the expression of this transcript in neoplastic tissue was not reported previously. Previous attempts to address this issue through sequence analysis of exon 1β in other tumor types revealed no mutations (14). In this report, we addressed the issue of possible β inactivation in breast cancer by performing SSCP analysis of the exon 1 region of β transcripts in all 23 tumors for which we obtained expression data and found no evidence of mutation in any of the tumors (data not shown). Although our own previous analysis (4) of exon 2 in breast tumors revealed three mutations of 21 tumors affecting the amino acid sequence for the β transcript (CGA-GGA, codon 87; GCA-GTA, codon 96; and CGC-CAC, codon 161), only one of these mutations was found in a region conserved in both mice and humans, and another was a frequently reported polymorphism. Furthermore, we have now shown that apparent loss of expression of the β ($p19^{ARF}$) transcript is primarily observed in those breast tumors in which $p16^{INK4a}$ expression is compromised. Taken together, this information indicates that point mutation or loss of expression is not common for the β transcript, and that there is no evidence to suggest a tumor-suppressive role for the β transcript in breast carcinogenesis. However, the reason for and possible conse-

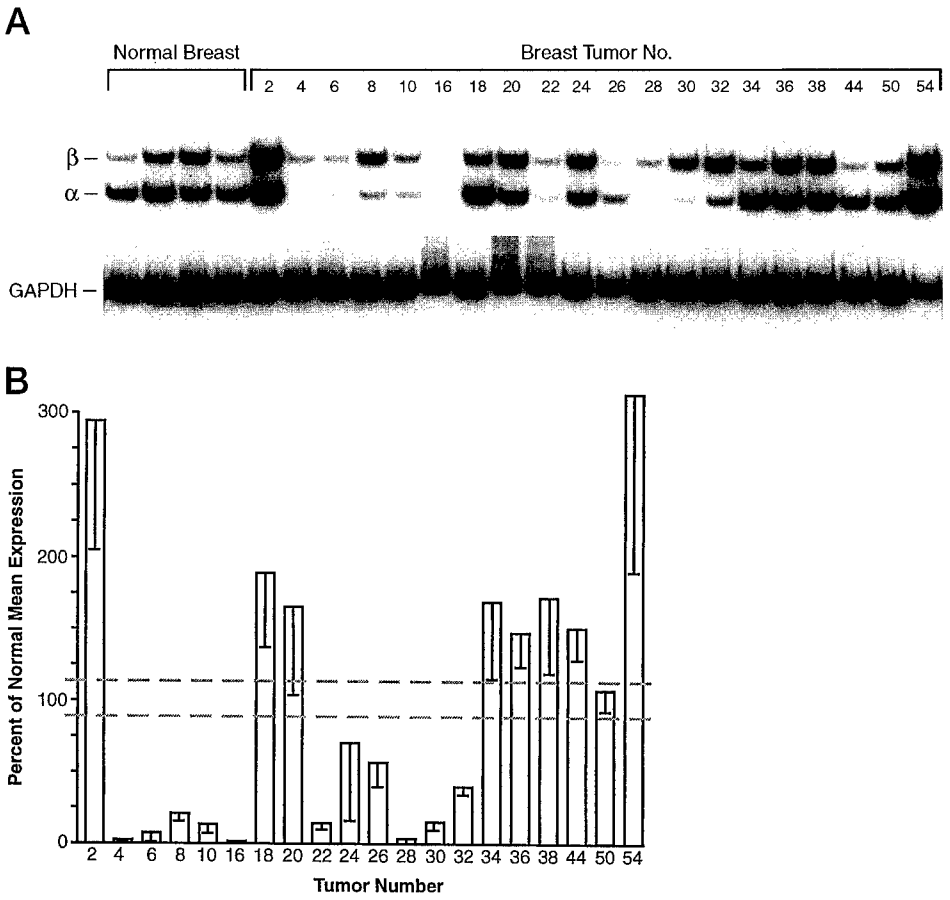

Fig. 3 Expression analysis of the α ($p16^{INK4a}$) and β ($p19^{ARF}$) transcripts in breast cancer by RT-PCR. *A*, autoradiograph of a representative analysis of α and β transcripts in 4 normal breast samples and 20 carcinomas of the breast. *B*, GAPDH-normalized expression levels of the α transcript as determined by triplicate analysis in 20 tumors (see text). *Dashed line*, normal mean SD; *bar*, sample SD.

quences of the observed overexpression of the β transcript in some breast tumors are unclear. Further experiments are needed to address these issues.

Nevertheless, comprehensive analysis of homozygous and hemizygous deletion, methylation, mutation, and expression suggest that the tumor suppressor $p16^{INK4a}$ is cumulatively affected in approximately 40–50% of the breast carcinomas analyzed. This rate of inactivation of $p16^{INK4a}$ and lack of inactivation of the β transcript implicate $p16^{INK4a}$ involvement in the tumorigenesis of the breast at a rate greater than or equal to that reported previously for any other tumor suppressor gene in sporadic breast cancer.

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Increased *p16* expression with first senescence arrest in human mammary epithelial cells and extended growth capacity with *p16* inactivation

Andrew J Brenner[1], Martha R Stampfer[2] and C Marcelo Aldaz[1]

[1]*Department of Carcinogenesis, The University of Texas MD Anderson Cancer Center, Research Division, Smithville, Texas 78957 and* [2]*Berkeley National Laboratory, Berkeley, California 94720, USA*

Aberrations affecting the tumor suppressor gene *p16*^{INK4a} have been described for a variety of tumors. In breast cancer, approximately 50% of tumors show low or lack *p16* expression. While evidence provided by some studies has implicated a possible role for *p16* in normal replicative senescence, other studies have suggested that the Rb, pathway through which p16 functions, may not be involved in senescence control. Previously we observed that all immortal lines derived from normal mammary epithelium which were analysed for *p16* displayed inactivation of this gene through distinct mechanisms, supporting *p16* inactivation as a possible necessary event in escape from senescence. To further clarify this issue, we have analysed p16 expression in a panel of normal finite lifespan human mammary epithelial cells (HMEC) from initial propagation through growth arrest, using media which confer different replicative capacity. Approximately 10–25-fold increase in *p16* expression was observed for all normal HMEC with initial onset of a senescence phenotype following 15–25 population doublings in culture. These cells also displayed expression of the senescence associated β-galactosidase. Interestingly, HMEC with additional long term replicative capacity (approximately 80 population doublings) arose from these growth arrested cultures, showing lack of *p16* expression. This extended growth capacity appears to be associated with a methylation phenomenon since treatment of these cells with the methylation inhibitor 5-aza-2-deoxycytidine resulted in growth arrest concurrent with reacquisition of p16 expression and senescence associated β-galactosidase. Analysis of *p21waf1* expression revealed no change in expression during growth *in vitro*. These results support *p16*^{INK4a} as the 9p senescence gene and suggest a role for *p16* loss in the escape from initial onset of senescence and in acquisition of an extended life span of human mammary epithelial cells.

Keywords: p16^{INK4a}; p21^{WAF1}; immortal; extended life; breast

Introduction

The p16$_{INK4a}$ protein, hereafter referred to as p16, is a known negative regulator of cell cycle progression through its cyclin dependent kinase (CDK) inhibiting function (Serrano *et al.*, 1993). p16 competes with the activating D type cyclins for association with CDK4 or CDK6, thereby preventing phosphorylation of proteins controlling G1 exit such as the retinoblastoma (Rb) protein (Sherr, 1996). Inactivation of *p16* has been observed in numerous tumor types (Merlo *et al.*, 1995; Gonzalez-Zulueta *et al.*, 1995; Herman *et al.*, 1995; Dreyling *et al.*, 1995; Xiao *et al.*, 1995a,b), and lack or reduced expression of *p16* has also been shown through a variety of technical approaches to occur in at least 50% of the breast cancer samples examined (Brenner *et al.*, 1996; Geradts *et al.*, 1995). While these findings suggest that *p16* may play an important role in breast tumorigenesis, the consequence of such aberrations of *p16* are not yet clear.

Of the putative functions of *p16*, the role as a candidate senescence gene is supported by several observations. The mapping of *p16* to chromosomal subregion 9p21, a region containing a putative senescence gene, is significant in this regard (Barrett *et al.*, 1994). Also, p16 is an upstream negative regulator of retinoblastoma protein phosphorylation (Serrano *et al.*, 1993). Studies by Stein *et al.* (1990) have shown that replicative fibroblasts phosphorylate Rb upon stimulation with serum following serum starvation, but in senescent fibroblasts, Rb remains unphosphorylated. Analogously, elevated p16 levels are seen in senescent fibroblasts as compared to young fibroblasts, suggesting that Rb remains unphosphorylated in senescent cells due to high levels of p16 (Barrett *et al.*, 1994; Alcorta *et al.*, 1996). These and other observations suggest *p16* may be important in the control of replicative senescence.

Although these observations support a putative role for *p16* in the senescence control of fibroblasts, the situation in human mammary epithelial cells has remained less clear. The human papilloma virus 16 (HPV) proteins E6 and E7, which interact with p53 and Rb respectively, are both required for the HPV mediated escape from senescence in fibroblasts leading to immortalization (Shay *et al.*, 1993a). However, in human mammary epithelial cells, the presence of E6 alone has been found to be sufficient for escape from senescence and the induction of an extended replicative capacity leading to immortalization (Band *et al.*, 1991; Shay *et al.*, 1993a). Further, mammary epithelial cells from patients with Li–Fraumeni Syndrome (germline mutation of p53) spontaneously immortalize *in vitro*, while stromal cells from these patients do not (Shay *et al.*, 1995). Normal human mammary epithelial cells have also been shown to undergo immortalization when transfected with only a mutant *p53* (Gollahon and Shay, 1996). This has led to the suggestion that, unlike in fibroblasts, the Rb pathway through which p16 exerts its growth suppressive effect is not involved in senescence control of mammary epithelial cells (Shay *et al.*, 1993a,b).

Correspondence: CM Aldaz
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However, in more recent studies, it has been suggested that distinct susceptibility of human mammary epithelial cells to E6/E7 induced escape from senescence may be dependent upon the stage of culture (Wazer *et al.*, 1995). While escape from senescence has been achieved by HPV 16 E6 alone, this was only seen in cultures with long term growth potential which had emerged from a period of growth arrest termed 'selection'. In contrast, most early passage cells which had not yet entered selection were immortalized by E7 alone and not E6 (Wazer *et al.*, 1995). The difference between these preselection and postselection cell populations which results in distinct susceptibility to viral immortalization has not been clear. Further, the reasons cells arrest growth at this stage, as well as the means of spontaneous escape, have not been elucidated.

We previously observed the inactivation of *p16* in immortal breast epithelial lines derived from normal mammary epithelium through three distinct mechanisms; homozygous deletion (MCF10 and MCF12 cell lines) nonsense mutation with hemizygous loss (184 A1 cell line), or hypermethylation (184 B5 cell line) (Brenner and Aldaz, 1995; and unpublished data). These findings supported p16 inactivation as a possible necessary event for escaping senescence. In this report, we sought to further characterize the role of *p16* in senescence control of human mammary cells. Normal primary human mammary epithelial cultures (HMEC) were followed from initial propagation *in vitro* until growth arrest and subsequent extended replicative growth. Expression analysis of *p16*, as well as another candidate senescence control gene $p21^{WAF1}$, was performed at various time points. The HMEC were also monitored for replicative capacity and expression of the senescence associated β-galactosidase.

Results

Analysis of p16 *in normal HMEC*

Normal HMEC *in vitro*, like normal fibroblasts, undergo a limited number of cell divisions (Stampfer and Yaswen, 1994). In the case of HMEC, the total number of population doublings which can be achieved, as well as other characteristics, has been shown to be dependent upon the type of media used to propagate the cells (Stampfer and Yaswen, 1994). Specifically, finite lifespan HMEC grown in the serum containing medium, MM, display active growth for 2–5 passages or 15–25 population doublings, with gradual loss of proliferative activity. The senescent population retains the typical epithelial morphology (Stampfer, 1985). In contrast, when HMEC are grown in the serum-free MCDB 170 medium, after active proliferation for 2–3 passages, almost all the cells cease growth, becoming large, flat, striated, with irregular cell borders (Stampfer, 1985; Hammond *et al.*, 1984). Following 2–4 weeks of inactivity, the population then undergoes a process termed 'self-selection' (Hammond *et al.*, 1984). There is active proliferation of small cells with the typical epithelial cobblestone morphology, which soon dominate the culture. These post selection cells maintain growth for an additional 7–24 passages (approximately 45–100 population doublings in total), after which flatter and more vacuolated cells appear which retain the epithelial morphology without further growth (final arrest).

To assess the possible role of p16 in HMEC senescence, HMEC from five different reduction mammoplasty specimens were cultured in both MM and MCDB 170 and followed from initial propagation through cessation of growth. Initially, the levels of *p16* were low in both culture conditions. These levels rose with increasing passage until initial growth arrest, when *p16* levels were 10–25-fold higher than original values (Figures 1a and b and 2). In contrast, assay of the emergent post-selection cells in MCDB 170 indicated that the level of *p16* transcript was drastically reduced (Figures 1c and d and 2). None of the post-selection HMEC revealed expression of *p16* during the following growth period, nor at the final growth arrest. These findings were confirmed at the protein level as well. Figure 3 shows an immunoblotting analysis of the 184 HMEC culture using an anti-p16 antibody. Cells grown in serum containing media (MM) start with low levels of p16 protein expression (Figure 3 MM passage 2) which increases and remains high until final growth arrest (Figure 3 MM passage 6). On the other hand and as observed with the p16 transcript analyses, when HMEC are grown in serum-free MCDB170 medium, p16 protein is detected in the pre-selection cells (Figure 3, 170, passage 2) but not in the post-selection cell population (Figure 3, 170, passage 20).

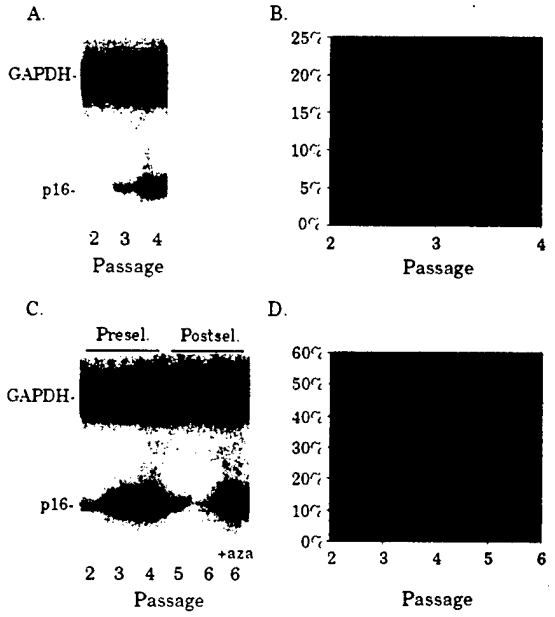

Figure 1 Expression of p16 in normal human mammary epithelial culture B26 from early culture through selection. (**a**, **b**) Duplex RT–PCR analysis of B26 cells grown in media MM reveals an increase in p16 expression relative to glyceraldehyde 6-phosphate dehydrogenase (GAPDH) expression with increased passage. Highest expression at P4 coincided with growth arrest. (**c**, **d**) Analysis of B26 grown in media MCDB 170 reveals a similar increase in relative expression. However, following growth arrest at P4, small cells emerge which apparently lack expression and soon dominate the culture. When treated with 3 μM 5-aza-deoxyctidine (aza) for 72 h to inhibit methylation, cells arrest growth and p16 levels rebound to those observed at P4

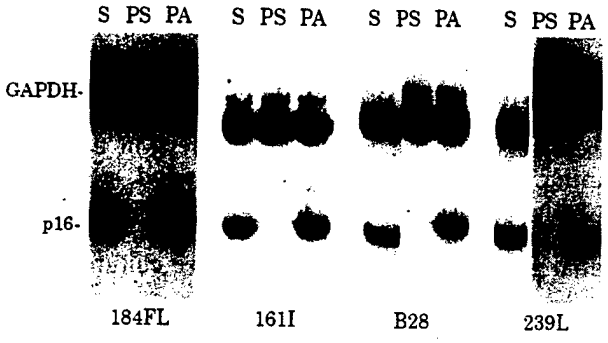

Figure 2 Duplex RT–PCR analysis of p16 expression relative to glyceraldehyde 6-phosphate dehydrogenase (GAPDH) expression in four additional normal human mammary epithelial cultures at initial growth arrest (S), post-growth arrest (PS), and when treated with a methylation inhibitor post-growth arrest (PA). Notice all cultures express high levels of p16 at initial growth arrest, but not following unless treated with a methylation inhibitor

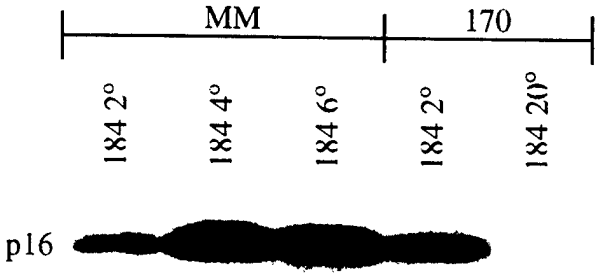

Figure 3 Immunoblot analysis of p16 protein expression in normal 184 human mammary epithelial cells. Early in culture, cells grown in serum containing media (MM) show low levels of p16 protein expression (MM passage 2) which increases in subsequent passages and remains high until final growth arrest (MM passage 6). The same HMEC grown in serum-free MCDB170 medium (170) show detectable p16 protein only in pre-selection cells (170, passage 2) but not in post-selection cells (170, passage 20). All lanes were control for equal protein loading (not shown)

Methylation of post-selection HMEC

Previous analysis of tumor cell lines as well as numerous primary tumors has shown inactivation of *p16* through de novo methylation of the promoter region (Herman *et al.*, 1995). Promoters silenced by methylation can be reactivated in many cases by treatment with 5-aza-2′-deoxycytidine, a drug which is an established inhibitor of DNA methylation. In order to determine whether cells arising after initial arrest in MCDB 170 lacked expression due to methylation, post selection cells were grown in the presence of 5-aza-2-deoxycytidine and evaluated for *p16* expression. Of the five post-selection cultures examined, all regained *p16* expression following treatment (Figure 1c; samples labeled PA, Figure 2). Further, analysis of the 184 post-selection cells by methylation-sensitive endonuclease digestion of DNA followed by Southern hybridization with a *p16* exon 1 probe, showed patterns consistent with complete methylation of *p16*, corroborating the aforementioned results (not shown). These data indicate that those cells capable of post-selection growth were inactive in *p16* expression due to de novo mthylation.

Coincidence of expression of p16 and senescence associated β-galactosidase

In analysis of senescent cultures, Dimri *et al.* (1995) observed the expression of an unusual endogenous β-galactosidase with a pH optimum of 6.0. This form of β-galactosidase was expressed by senescent cells *in vivo* as well as those cultured *in vitro*, but not by presenescent, quiescent, or terminally differentiated cells. While the source or function of this novel β-galactosidase is unknown, it nevertheless constitutes a useful senescence associated marker. To establish whether this marker showed coincident expression with growth arrest and increased *p16* expression of the HMEC grown in both culture conditions, cells were evaluated at various time points for the presence of senescence-associated β-galactosidase (SA β-Gal).

As expected, analysis of SA β-Gal in the early actively growing HMEC in either MM or MCDB 170 revealed no activity. As previously mentioned, these same cell populations also showed low *p16* expression. However, when cells ceased replicating following the 2nd–5th passage in both media, high levels of SA β-Gal were displayed, coincident with the highest levels of *p16* (Figure 4a and b). In contrast, the newly emergent post-selection HMEC from the same cultures in MCDB 170, which had no *p16* expression due to apparent hypermethylation of the *p16* promoter, did not display SA β-Gal activity positive cells. When these actively growing cells were treated with 5-aza-2-deoxycytidine to reverse methylation, cells ceased growing, regained expression of *p16* (as mentioned above), and expression of SA β-Gal was again observed concurrently (Figure 4e).

Analysis of $p21^{WAF1}$ expression with senescence

Another CDK inhibitor, $p21^{WAF1}$, has been shown to be induced during senescence in fibroblasts (Alcorta *et al.*, 1996; Levine, 1997). In order to determine whether *p21* expression is associated with senescence of HMEC, levels of *p21* expression were followed in the aforementioned cultures. High levels of *p21* were observed during initial propagation and showed little variability during growth arrest or extended replicative life (Figure 5). Thus, while increased expression of *p21* was seen with senescence in some cell types, this does not appear to be the case with HMEC, suggesting that *p21* does not play as significant a role in senescence of HMEC as $p16^{INK4a}$. This is similar to observations in adrenocortical cells, which express high levels of $p21^{WAF1}$ throughout their replicative life span to senescence (Yang *et al.*, 1995).

Discussion

As previously mentioned, the number of population doublings which can be achieved in the culture of HMEC has been shown to be largely dependent upon

the type of culture media used (Stampfer and Yaswen, 1994). Nevertheless, all HMEC undergo a period of initial growth arrest at an approximately similar level of growth, from which only those cells grown in serum free media recover. The reason cells undergo this initial growth arrest and the mechanisms by which cells recover from it, have not been understood. In the series of HMEC examined in this report, all showed an increase in *p16* expression with progression toward initial growth arrest, at which point the highest levels of *p16* were observed. When grown in serum free media, foci of actively growing cells emerged from these seemingly senescent cultures at a low frequency ($\sim 10^{-5}$) and were devoid of *p16* expression due to possible hypermethylation of the *p16* promoter region. These cells did not regain *p16* expression at anytime during their remaining replicative period, nor at final growth arrest (Figure 3). These data would suggest that increased expression of p16 may be causative of the initial growth arrest observed in these cultures.

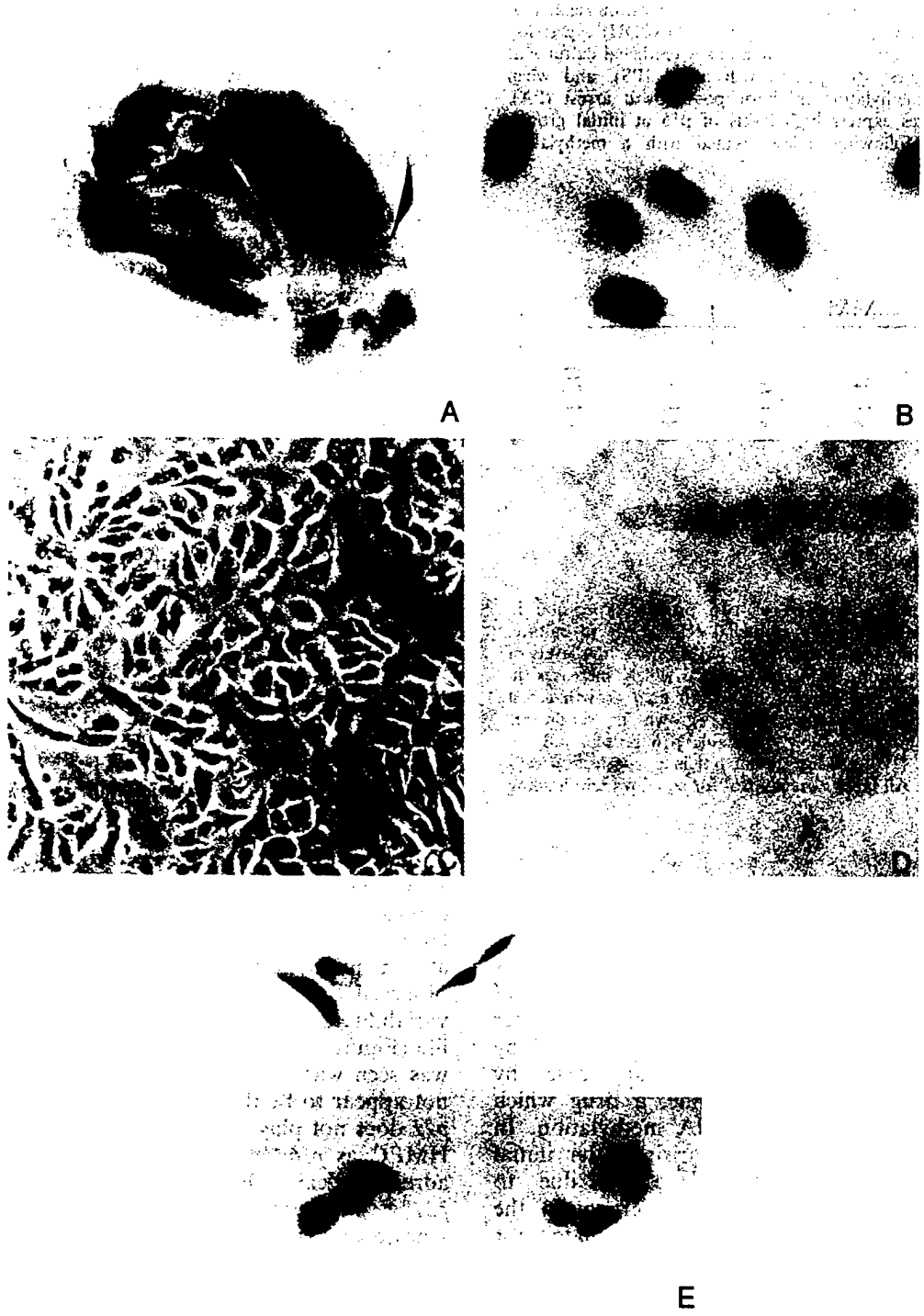

Figure 4 Analysis of senescent associated beta-galactosidase (β-gal) in normal human mammary epithelial cultures. **(a)** Representative β-gal staining from a terminally arrested culture in media MM (bright phase). Culture shown is B26 at passage 4. **(b)** Representative β-gal staining of a first arrest culture in media MCDB 170 (bright phase). (c) Post-selection cells (phase contrast), culture 184. **(d)** Same field as c (bright phase). (e) Post selection cells treated with 3.3 μM 5-aza-2-dexycytidine for 72 h, culture 184

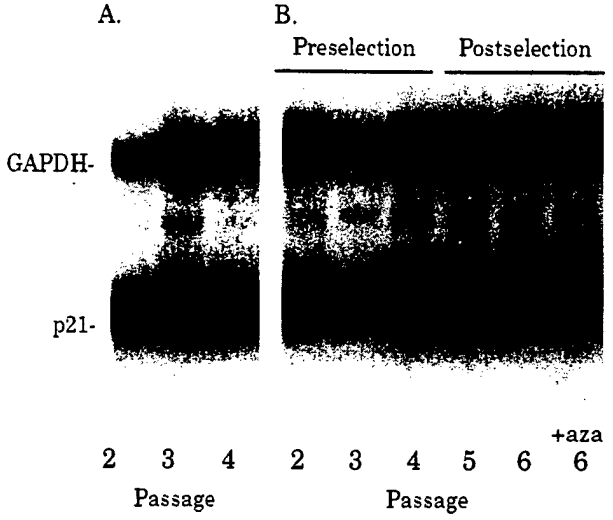

Figure 5 Expression of p21 in normal human mammary epithelial culture B26 from early culture through selection. (a) Duplex RT–PCR analysis of B26 grown in media MM reveals little relative change in p21 expression relative to glyceraldehyde 6-phosphate dehydrogenase (GAPDH) expression with increased passage. (b) Analysis of B26 grown in media MCDB 170 reveals a similar lack of relative change in expression, even when treated with 3.3 μM 5-aza-2-dexycytidine (aza) for 72 h

It has been suggested (Foster and Galloway, 1996) that initial growth arrest of HMEC may be indicative of a normal senescence and an early mortality stage which is distinct from those previously postulated in the Shay *et al.* (1993b) two stage model of human mammary epithelial immortalization. Indeed, the increased expression of *p16* in fibroblasts with onset of senescence has been previously described (Alcorta *et al.*, 1996), and we now show a similar increase with the onset of initial growth arrest in HMEC. Additionally, our finding of expression of senescence marker SA β-Gal in cells at this initial growth arrest, as well as in those post selection cells growth arrested with regain of p16 expression when treated with a methylation inhibitor, adds weight to this prospect. Nevertheless, while SA β-Gal may be the best marker available for determination of senescence, it is not an absolute indicator. Further, SA β-Gal was also observed during the final growth arrest which has classically been viewed as senescence. Additional studies are needed to better address how the initial arrest and final arrest may relate to senescence *in vivo*.

As previously mentioned, analysis of immortalization of human mammary epithelial cells by E6 and E7 genes has shown susceptibilities distinct to those found in fibroblasts (Shay *et al.*, 1993a). Therefore, it has been proposed that while both Rb and p53 are involved in fibroblast immortalization, only p53 appeared to play a role in mammary epithelial cells (Shay *et al.*, 1993a,b). However, it is worth noting that the analysis of susceptibility to immortalization by E6 and E7 was performed with 'post-selection' mammary epithelial cells grown in media MCDB 170, which we have now shown to lack *p16* expression (Shay *et al.*, 1993a). This would suggest that the lack of necessity for E7 in immortalization of mammary epithelial cultures is due to prior inactivation of *p16*, and subsequent alteration of the Rb pathway. In such circumstances, the sequestration of Rb protein by E7 may confer no known additional benefit. Further, it suggests that similar to fibroblasts, both Rb and p53 dependent pathways play a role in the senescence control of mammary epithelial cells.

In support of this argument, recent studies by Wazer *et al.* (1995) have revealed that mammary epithelial cells present in milk and grown in a high serum supplemented media, similar to cells grown in serum supplemented media MM which we show to maintain *p16* expression, show substantial extension of life span with E7. While E6 was not able to confer a similar extension of life span in these cultures, its presence was required for complete immortalization. Additionally, in similar analysis of susceptibility to E6 and E7 immortalization of pre-selection and post-selection mammary epithelial cultures from tissue grown in media DFCI-1, a medium of constitution similar to MCDB 170 which results in selection, Wazer *et al.* (1995) were able to demonstrate that most pre-selection HMEC cultures undergo extension of lifespan followed by immortalization with E7 and not E6. In contrast, post-selection HMEC immortalize with E6 and not E7. This was subsequently corroborated by others, and the arrest which E7 is capable of circumventing was designated M0 by Foster and Galloway (1996) to conform to nomenclature previously used in a two stage model of immortalization suggested by Shay *et al.* (1993a). Thus, the requirement for E7 in immortalization of cells expressing *p16* (pre-selection), and lack of necessity for E7 in the immortalization of mammary epithelial cells with inactivated *p16* (post-selection), supports the role of *p16* as a senescence control gene in human mammary epithelial cells. Additionally, this suggests that a chronology exists in the senescence control of human mammary epithelial cells where increased p16 expression may be involved in an initial stage (M0) of normal growth arrest. Inactivation of this initial control stage (e.g. p16 inactivation or Rb sequestration by E7) appears to result in a limited increase in replicative capacity and suspectibility to further extension of lifespan by circumvention of the second (M1) stage (e.g. by E6 or mutant p53 (Shay *et al.*, 1993a). Following a final period of selection (crisis, M2) immortal cells emerge. This may by typified by previous experiments such as those of spontaneous immortalization of post-selection (grown in MCDB 170) mammary epithelial cells from Li–Fraumeni patients, while fibroblasts from these same patients required E7 to immortalize (Shay, 1995). Adaptation of previously suggested two stage models of mammary epithelial cell immortalization to include a first step dependent upon Rb pathway inactivation might be necessary.

In conclusion, in this report we show an increase in expression of *p16* with HMEC growth *in vitro*, the highest levels of *p16* expression coinciding with the first growth arrest and expression of a senescence marker, SA β-Gal. Cells emerged from these seemingly senescent cultures devoid of *p16* expression possible due to hypermethylation of the *p16* promoter region. No change was seen in $p21^{WAF1}$ expression from initial propagation through the entire culture period. These results support *p16* as well as the Rb pathway in the senescence control of normal mammary epithelial cells.

Further, these results provide insight into the possible reason for differences between stromal and epithelial cells observed in previous studies of induced extended life utilizing cells which have undergone 'selection' in serum free media.

Materials and methods

Cell culture

Normal human breast tissue specimens from reduction mammoplasties were obtained and epithelial organoids were separated from stromal components as previously described (Stampfer *et al.*, 1980). Organoids were cultured in parallel in either a complex serum containing medium, MM (Stampfer, 1982, 1985), containing 0.5% fetal bovine serum, conditioned media from the cell line Hs767B1 (30%), insulin (10 μg/ml), hydrocortisone (0.1 μg/ml), epidermal growth factor (5 ng/ml), and cholera toxin (1 ng/ml), or in the serum free medium MCDB 170 (23,24) supplemented with insulin (5 μg/ml), hydrocortisone (0.14 μM), epidermal growth factor (10 ng/ml), transferrin (5 μg/ml), isoproterenol (1 μM) and bovine pituitary extract (70 μg/ml).

For analysis of methylation, 5-aza-2′-deoxycytidine was added to a final concentration of 3.3 μM for 72–96 h.

DNA and RNA extraction

Total genomic DNA was isolated using phenol : chloroform : isoamyl alcohol (25 : 24 : 1) in Phase Lock Gel tubes (5 Prime→3 Prime, Boulder, CO), according to standard protocol, and precipitated with 2.5 volumes ethanol. Total RNA was isolated using an RNEasy Total RNA kit (Qiagen, Chatsworth, CA) as per manufacturers instruction.

Southern analysis for hypermethylation

Methylation analysis was performed as previously described (Brenner *et al.*, 1996). Briefly, 10 μg of genomic DNA were digested with a flanking site enzyme (either *Eco*RI or *Hind*III) and a methylation sensitive endonuclease (*Sac*II, *Sma*I and *Eag*I). The digested fragments were ethanol precipitated, resuspended and resolved in 1% agarose gel overnight. DNA was transferred to a Zeta-Probe nylon membrane (BioRad, Richmond, CA) and hybridized with a 340 bp or 280 bp α-^{32}P-dCTP random prime labeled PCR fragment including exon 1α, as described (Merlo *et al.*, 1995). Autoradiographs were obtained following 2–4 days of exposure.

Duplex RT–PCR

Five μg of total RNA were used for first strand cDNA synthesis with Superscript II reverse transcriptase (Gibco BRL, Gaithersburg, MD) as per manufacturers instructions. Following reverse transcription, 4 μl (1/15 of total) each sample was subjected to amplification with 200 μM each primer, 2.0 mM $MgCl_2$, 1 × Taq Buffer, 10% dimethylsulfoxide, 0.7 mM dNTPs, in a final volume of 20 μl. Following a 5 min denaturing at 96°C, samples were held at 90°C while five units of Taq DNA Polymerase (Promega) were added, followed by 20 cycles at 94°C, 50°C, and 72°C for 40 s each. Reaction products were diluted 50-fold, and subjected to an additional 13 cycles (*p16*) or ten cycles (p21) of amplification utilizing one nested ^{32}P-polynucleotide kinase end labeled primer per reaction product, employing the same conditions as the first amplification. Reaction products were resolved on 5% (1 : 30) native polyacrylamide, followed by autoradiography. Signal intensities in all cases were analysed and quantified with a Molecular Dynamics Phosphorimager.

Primers used were as follows: GAPDH 5′, CCGATGGCAAATTCGAT GGC; GAPDH 3′, GATGACCCTTTTGGCTCCCC; P16 5′, CAACGCACCGA ATAGTTACG; P16 3′(1), AGCACCACCAGCGTGTC; P16 3′(2), CGTGTCCA GGAAGCCC; GAPDH 3′(P21), CAGAGATGATGACCCTTTTGGC; P21 5′(1), CAGCAGGAAGACCATGGTG; P21 5′(2), GACCTGTCACTGTCTTGTACC; P21 3′, CCTGTGGGCGGATTAGGGCTTCC.

Immunoblot analysis

Protein expression analysis was performed using an anti-p16 antibody as previously described (Sandhu *et al.*, 1997).

SA B-Gal activity staining

Cells were washed 2 × with PBS, fixed in neutral buffered formalin, and stained as previously described (Dimri *et al.*, 1995). Briefly, following fixation, cells were incubated overnight at 37°C in a reaction buffer containing X-gal (1 mg/ml), 40 mM citric acid/sodium phosphate (pH 6.0), potassium ferrocyanide/ferricyanide (5 mM), NaCl (150 mM) and 2 mM $MgCl_2$. Negative activity control was breast carcinoma line T47D and positive activity control were senescent diploid fibroblasts.

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THE MOLECULAR BASIS OF BREAST CARCINOGENESIS

April Charpentier, Ph.D. and C. Marcelo Aldaz, M.D.

Department of Carcinogenesis, The University of Texas M.D. Cancer Center,

Science Park - Research Division, Smithville, Texas 78957

Tele: (512) 237-9530

Fax: (512) 237-2475

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INTRODUCTION

Breast cancer is the most common malignancy affecting women today. This disease has reached epidemic proportions in the industrialized world afflicting as many as one in eight women (1), and causing approximately 45,000 deaths per year (2,3). In response to this major public health problem, research funding is being used to identify key steps in breast carcinogenesis with the goal of developing effective means for preventing, diagnosing and treating this devastating disease.

Overwhelming evidence has accumulated indicating that breast cancer is a genetically-based disease in which spontaneous mutation and/or hereditary genetic predisposition play primary roles. Environmental and epigenetic influences are also important, however their contribution to breast carcinogenesis is, at present, not well understood. The potential for developing breast cancer is most likely determined by the specific genetic makeup of an individual woman, however, this potential only becomes a threat by the interaction with specific endogenous as well as, perhaps, exogenous factors (Figure 1). For example, an important endogenous risk factor would include the extent of exposure to a woman's own ovarian hormones (4). Early age at menarche and nulliparity have been linked to an increased risk for developing breast cancer. It is likely that the extent of endogenous hormone exposure is genetically pre-determined or at least influenced by genetic factors. Examples of exogenous factors would include lifestyle choices such as, diet, alcohol intake and cigarette smoking (Figure 1). Physical environmental causes, such as ionizing radiation, have been shown to increase the likelihood of breast cancer development (5,6). Also, although

the topic is controversial and less understood, we should mention the potential role of environmental pollutants and hormone disrupters (7) (8). Taken together, it is then the combination of the genetic constitution, plus the influence of multiple endogenous and exogenous factors which, ultimately, will determine the overall risk of any particular woman for developing breast cancer.

Numerous mutated genes have been shown to be linked to breast cancer development although the assumption is that there are many more important breast cancer genes yet undiscovered. This overview focuses on the currently identified genes as well as genetic aberrations which may lead to the identification of as yet unknown genes key to breast carcinogenesis. In addition, other factors which also exercise significant influence in breast carcinogenesis, such as the hormonal regulation of cell proliferation, are discussed.

HISTOPATHOLOGY

Normal Breast Tissue

In order to correlate the genetics of breast cancer to the clinical manifestations, an overview of the histology of both normal and cancerous breast tissue is required. It should be mentioned, however, that it is beyond the scope of this chapter to perform a detailed analysis of breast tumor pathology.

The mammary glands are derived from modified sweat glands and basically represent downgrowths of the epidermis. In the adult woman the mammary gland is composed of approximately 15-20 lobes of branched

tubuloalveolar glands. Dense fibrous connective tissue separates the lobes. Each lobe in turn is subdivided into multiple lobules. Mammary lobules are located at the deepest end of the duct system. These lobules form clusters identified as blind-ending terminal ductules, or acini. These structures are embedded into a loose connective tissue rich in capillaries. Dense fibrocollagenous support mixed with abundant adipose tissue fills the interlobular spaces. The main collecting ducts (i.e. the lactiferous ducts) are lined by stratified squamous epithelium near their opening onto the nipple. A short distance from the surface each lactiferous duct presents a dilated portion known as the lactiferous sinus where the epithelial lining shows a transition to a two layer cuboidal epithelium. From there and throughout the rest of the duct system and acini the epithelium consists of one layer of luminal cuboidal cells and a basal layer of myoepithelial cells (Figure 2).

The development and differentiation of the mammary gland is hormonally regulated. The ovarian hormones estrogen and progesterone control breast development, especially during puberty. Full or complete differentiation of the mammary gland takes place during pregnancy and lactation, a time when the hormone prolactin plays a fundamental role. The explosive growth that the mammary epithelium undergoes during pregnancy causes the mammary tree to branch dramatically increasing the number of acini. This level of differentiation constitutes what Russo and Russo have described as lobule type 3 (9).

Wellings has suggested that the majority of breast carcinomas originate in what is known as the terminal ductal-lobular unit (TDLU) (10), also known as lobule type 1 (9). The TDLU has only 6-10 terminal ductules/lobule and is equivalent to the less differentiated state of

mammary gland development found predominantly in the breast of the nulliparous women (9).

Invasive Carcinoma

The most frequently observed invasive breast carcinoma is the infiltrating (invasive) ductal carcinoma (IDCA). IDCA represents approximately 75-80% of the total invasive breast cancer cases (11). Most invasive ductal carcinomas display the typical phenotype of well to poorly differentiated adenocarcinomas. Another type of invasive breast carcinoma, which accounts for approximately 10-15% of the total invasive breast cancer cases, is the infiltrating lobular carcinoma (ILCA) (Rosen, 1979). ILCA has a very distinctive infiltrating growth pattern, characterized by the pathognomonic presence of isolated cells or cord of cells (i.e. Indian files pattern) (11). This tumor type also presents a different clinical and metastatic pattern than the invasive ductal type (13). For example, patients with invasive lobular carcinoma have been reported to have a higher risk of developing multifocal and contralateral breast cancer than patients with invasive ductal carcinoma (14). It is important to remember, that the designation of ductal and lobular carcinomas does not imply that ductal carcinomas originate exclusively in ducts and lobular carcinomas in lobules. According to Wellings, both tumor types, ductal and lobular, appear to originate in the TDLU (10).

Preinvasive and Hyperplastic Lesions

In an attempt to clearly identify stages of breast cancer development, standardized terminology is used to refer to important changes in the morphology of the breast epithelium which have been noted by pathologists. This is shown schematically in Figure 3.

The identification and nomenclature of "potentially premalignant" lesions of the human breast have been a matter of controversy for many years. Among the non-invasive breast lesions, ductal carcinoma *in situ* (DCIS) is the most common and best characterized precursor to invasive carcinoma. Some researchers have also proposed models where there is a direct transition from normal to malignant epithelium, without any visible evidence of a preneoplastic stage (15). Nevertheless, evidence placing DCIS as a major precursor lesion of invasive carcinomas is substantial. For instance, the majority of IDCA's have an *in situ* component (16). Women with biopsy-proven DCIS have an increased risk for development of subsequent invasive breast cancer (17) and a high recurrence of invasive carcinoma occurs in women who have had breast-conserving treatment of DCIS (18). Taken together these findings strongly suggest *in situ* carcinomas are precursors to invasive carcinomas. A more insidious lesion is lobular carcinoma *in situ* (LCIS). LCIS is characterized by a uniform population of generally small and loosely cohesive cells growing in a solid occlusive fashion. Whereas the more common DCIS represents a highly heterogeneous group of lesions ranging from microscopic to grossly detectable intraductal carcinomas, as well as predominantly *in situ* carcinomas with areas of stromal microinvasion. When breast neoplastic cells begin proliferating outside their site of

origin, (i.e. beyond the containment of the basement membrane) and the microinvasion spreads further, the neoplasia is then termed invasive carcinoma. Invasive ductal carcinomas are not necessarily the result of DCIS nor are invasive lobular carcinomas the obligated direct result of LCIS. In fact, there is some evidence that LCIS may play an important role as a precursor of invasive ductal carcinoma as well (Figure 3) (19). It should be mentioned however that the relationship of LCIS as a precursor lesion of invasive breast cancer is a matter of controversy (19). It has been suggested that due to its morphologically diffuse and generalized nature, the presence of LCIS indicates that the whole breast epithelium is at risk of malignant transformation. It is possible that the distinction between DCIS or LCIS may be indicative of important differences in their genetic pathways during breast carcinogenesis.

Mammary epithelial hyperplastic lesions which demonstrate only some of the characteristics of *in situ* carcinoma are frequently identified in the clinic. These lesions are called atypical hyperplasias (AH) (20). Atypical hyperplasias can either be ductal (ADH) or lobular (ALH) in type. Finally, other less advanced hyperplastic lesions which are only associated with a slightly increased risk for breast cancer development, are known as proliferative disease without atypia (PDWA). PDWA lesions lack the qualitative and quantitative histologic features of AH (20) meaning that they comprise a variety of epithelial hyperplastic lesions from mild to florid but do not show signs of atypia.

FAMILIAL AND SPORADIC BREAST CANCER FORMS

Family history constitutes the strongest known risk factor for development of breast cancer. Women who have a family tree in which several blood relatives were afflicted with breast cancer have a far greater chance of developing breast cancer as compared to the general population. Because of this fact, in the last few years intense research has focused on identifying the breast cancer susceptibility genes, passed down from generation to generation. However, the inherited forms of breast cancer account for only 5-10% of total breast cancer incidence (Figure 4). The remaining 90-95% of women who develop breast cancer do not appear to segregate for an inherited susceptibility allele. Such cases are known as "sporadic" breast cancer (21). This label is somewhat misleading since "sporadic" breast cancer is actually the most frequent form of breast cancer. Nevertheless, approximately 25% of breast cancer cases diagnosed before age 30 are believed to be caused by genetic factors alone (22). Analysis of familial pedigrees suggested the existence of various types of inherited breast cancers, consistent with models of autosomal dominant transmission of a highly penetrant susceptibility allele (21). The major familial breast cancer forms include (23):

1.) site-specific breast cancer, which is the most frequent and occurs in families in the absence of any other familial occurring neoplasm;

2.) breast and ovarian cancer syndrome, which is characterized by early-onset and high rate of bilaterality;

3.) Li-Fraumeni cancer syndrome, which is characterized by early-onset of breast cancer, bilaterality, and association with other

familial neoplasias, such as leukemia, sarcomas, brain cancer and adrenocortical carcinoma;

4.) Cowden disease which is a rare condition also known as multiple hamartoma syndrome and is characterized by multiple mucocutaneous hamartomatous lesions, both benign and malignant.

In addition to the above forms the Muir Torre Syndrome which is a variant of Lynch II syndrome, also includes breast cancer, although it is a very rare syndrome. This syndrome is caused by mutations in DNA mismatch repair genes and is associated with microsatellite instability (24,25).

BREAST CANCER SUSCEPTIBILITY GENES

BRCA1

We have witnessed tremendous progress within recent years in the identification of genes responsible for several of the inherited breast cancer types. In 1990 genetic linkage analysis of affected families identified a gene predisposing individuals for early-onset breast cancer. This locus (i.e. Breast cancer 1 or BRCA1), was mapped to chromosome region 17q21 (26,27). Furthermore it was estimated that this tumor susceptibility allele, would account for 45% of families with high incidence of site-specific breast cancer and approximately 80% of families identified as carriers of the early-onset breast and ovarian cancer syndrome (28). After intense effort, the gene itself was cloned in

1994 by Miki and coworkers (29). Mutations in BRCA1 were found to co-segregate with the predisposing haplotype in affected kindred (29). Thus, a woman who has a mutation in the BRCA1 gene has a high risk of developing breast cancer and this risk increases over her lifetime reaching a peak by the age of 70 with a risk of 87% (28).

BRCA1 is a large protein with 22 exons and 1,863 amino acids and shows very little homology to other known genes and also has several alternative spliced forms. However, based on the fact that BRCA1 has a ring finger motif close to its amino-terminus and a leucine heptad repeat within its sequence, speculation was made that BRCA1 may function as a transcription factor (30). BRCA1 mutations are scattered throughout the entire coding region. Interestingly, a frequently found mutation (185delAG) is also found to be present in 1% of women from Ashkenazi Jewish descent (31). Most commonly the germinal mutations affecting BRCA1 are small insertions and deletions causing frameshifts, which produce stop codons, and result in truncation of the protein product.

The BRCA1 gene product appears to play a much smaller role, if any, in non-familial breast cancer. As with other tumor suppressor genes it was expected that mutations of BRCA1 would be frequent in sporadic breast cancer forms, particularly due to the common finding of 17q loss of heterozygosity in most breast tumors (32-35). However, no mutations in BRCA1 have been found in non-familial breast cancer cases. It has been suggested that subcellular mislocation of the BRCA protein may play a role in sporadic breast cancer. In normal breast epithelial cells, BRCA1 is localized in the nucleus, whereas in the majority of breast cancer cell lines and in malignant pleural effusions from breast cancer patients and in some primary tumors it is localized, mainly in the cytoplasm (36).

Some groups have suggested that BRCA1 is a secreted protein since it contains certain homology regions to granins, a protein found in secretory granules (37). Conflicting with this suggestion, the nuclear localization originally reported in normal cells was later confirmed by other groups (38). Some evidence has also accumulated indicating that normal BRCA1 may act as a tumor suppressor gene inhibiting tumor growth (26,39). At present the function of BRCA1 is controversial and therefore requires further analysis. However, both BRCA1 and BRCA2 have been shown to bind and colocalize in the nucleus with the DNA repair protein RAD51 but further analysis is needed to define BRCA's role in this pathway (38,40,41). Furthermore, it was recently suggested that BRCA1 is required for transcription coupled repair of oxidative damage (42). These investigators showed that cells deficient in BRCA1 are impaired in their ability to carry out transcription coupled repair of oxidative damage (42). This would imply that BRCA1 may be playing an important role as guardian of genomic integrity.

BRCA2

A second breast cancer susceptibility gene was isolated in 1995, named BRCA2 (43). BRCA2 was originally mapped by linkage analysis to chromosome arm 13q12-13 (44). Similar to BRCA1, BRCA2 is a large gene encoding for 3,418 aminoacids and has several splice variants. Germinal mutations on this gene predispose a person to early-onset, site specific breast cancer and moderately predispose a woman to ovarian cancer. These families also present a higher incidence of male breast cancer and are associated with a higher predisposition to prostate, pancreatic, colon and

other cancers (43). As with BRCA1, the germinal mutations identified are spread throughout the coding sequence of BRCA2. Most of the mutations were frameshift mutations generating a truncated gene product as was the case with BRCA1. A particular BRCA2 germinal mutation, in this case 6174delT, is also frequently found (1%) in Ashkenazi Jewish women (45). Like BRCA1, BRCA2 appears to play no major role in sporadic breast cancer, since only very few somatic mutations were observed in these tumors. The function of the BRCA2 protein is uncertain as well.

Very recently the contribution of BRCA1 and BRCA2 to inherited breast cancer was assessed by linkage and mutation analysis in a series of 237 families with a history of breast cancer chosen at random without regard to the existence of other cancers (46). Linkage to BRCA1 was observed in 52% of the families, to BRCA2 in 32% and to neither in 16% of the families, indicating the existence of other predisposing genes. The vast majority (81%) of the breast/ovarian cancer families were associated with BRCA1 mutation, 14% due to BRCA2, while 76% of families with both female and male cancer cases were due to BRCA2. BRCA2 carriers appear to have a similar lifetime cancer risk as BRCA1 carriers but a lower risk before age 50 (46).

TP53

Germline TP53 mutations have been found in affected families and shown to be causative of the Li-Fraumeni cancer predisposition syndrome (47,48). Breast cancer is one of the neoplasms affecting patients with this syndrome. In tumors from patients with Li-Fraumeni syndrome, loss of the wild-type TP53 allele is observed with retention of the mutant

allele. As indicated above, this syndrome is characterized by early-onset breast cancer, bilaterality, and association with other familial neoplasias, such as leukemia, soft tissue sarcomas, osteosarcoma, brain cancer and adrenocortical carcinoma.

Contrary to the previously described tumor susceptibility genes, TP53, a known tumor suppressor gene, has been shown to play an important role in sporadic breast cancer progression as well. However, germline mutations of this gene in the general population are rare. TP53, located on chromosome arm 17p13, is known to harbor somatic mutation in 25-45% of primary breast carcinomas (49). All evidence indicates that TP53 is one of the most frequently affected genes in breast cancer. The role of TP53 in breast carcinogenesis will be further discussed in a following section.

PTEN / MMAC1

Recently, a new putative tumor suppressor gene, PTEN, has been identified on chromosome 10q23.3. PTEN is responsible for Cowden disease's familial predisposition (50-52). Breast cancer is a component of this rare syndrome as was described in a preceding section. The majority of the women who have this mutated gene develop breast neoplasia and approximately half of these cases develop into breast cancer. The amino acid sequence of PTEN resembles two different types of proteins: tyrosine phosphatases, enzymes that remove phosphate groups from the amino acid tyrosine in other proteins; and tensin, a protein that helps connect the cell's internal skeleton of protein filaments to its external environment (53). Homozygous deletions and mutations affecting

PTEN have been found in both prostate and glioblastoma cancer cell lines. Somatic inactivating mutations of PTEN were associated with numerous primary prostate and endometrial carcinomas (54,55). However, practically no somatic mutations affecting PTEN where found in sporadic forms of breast cancer (56,57).

ATM and HRAS (Putative breast cancer susceptibility genes)

Several years ago it was suggested that heterozygous carriers of a defective Ataxia-telangiectasia (ATM) gene are at increased risk (3-5 fold) of developing breast cancer (58). However, later studies concluded that there was no clear association between the ATM mutants and the risk of early onset or familial breast cancer in general (59,60). Nevertheless, it is an isue of importance since ATM heterozygous carriers represent a high percentage of the population (0.5% - 5%). At this point, the role of ATM as a factor for increasing breast cancer risk in the general population is unclear.

Other potential breast cancer susceptibility alleles are the polymorphic variants of the HRAS gene minisatellite sequence (61,62). These studies reported a positive association between rare HRAS alleles and breast cancer (61,62). However, controversial results have been reported by other research groups (63) and further confirmation of the positive association between these rare alleles with breast cancer is required.

Common Enzymes Allelic Variants that may Contribute to Breast Cancer Risk

Through molecular epidemiology studies, it is becoming apparent that the combination of a specific genetic makeup plus exposure to specific exogenous factors (e.g. environmental, chemical and physical carcinogens) play decisive roles in defining the risk for tumor development. The heterogeneity of the genetic background found in the general population would explain why certain individuals develop cancer while others do not even when exposed to a similar dose of a particular carcinogen (e.g. cigarette smoke and lung cancer risk).

Therefore, as previously suggested, breast cancer etiology may be explained by inherited predisposition to develop cancer, inherited predisposition to accumulate new mutations, and exogenous exposures (64) (Figure 1). This would be the basis for the expected etiologic heterogeneity found in the general population. Thus, although breast cancers are classified as a single disease, not all are caused by the same set of etiologic agents. Most likely, different population subgroups will respond differently to the same set of carcinogens.

Numerous studies have focused on a series of allelic variants that would confer increased tumor susceptibility. Several of these genes are enzymes involved in detoxification pathways that the organism utilizes to eliminate xenobiotics. In most cases, a phenotypic polymorphism in the metabolic rate of specific chemicals correlated with the finding of genotypic polymorphisms (i.e. allelic variants). The genotypic polymorphisms were detectable as restriction fragment length polymorphism (RFLP's) variants or as gene deletions. A good example of

such polymorphism affecting breast cancer is seen with the Cytochrome P-450 (CYP) superfamily of enzymes. These "Phase I" enzymes are responsible for the oxidative metabolism of diverse endogenous and exogenous substrates, such as steroids, prostaglandins, fatty acids, foreign chemicals and drugs. CYP enzymes are responsible for the biotransformation of xenobiotics to toxic intermediate metabolites (i.e. phase I). The level of CYP expression and CYP's catalytic activity can vary dramatically among the general population due to the highly polymorphic nature of these enzymes. The "Phase II" group of detoxification enzymes are also usually polymorphic and are responsible for the conjugation reaction necessary for the efficient excretion of toxic compounds. These enzymes transform toxic compounds into more hydrophilic forms for excretion. Ambrosone and Shields (65) have suggested that women who have genetic polymorphisms that could result in greater activation, or impaired detoxification of; aromatic and heterocyclic amines, (e. g. NAT1, NAT2, CYP1A2 enzymes); polycyclic aromatic hydrocarbons (e. g. GSTM1, CYP1A1 enzymes); and nitroso compounds (e. g. CYP2E1 enzyme) may be at greater risk for developing breast cancer. Recently it was reported that the GSTM1 homozygous null phenotype was associated with increased risk of developing breast cancer, similar associations were also observed with polymorphic variants of the enzymes GSTT1 and GSTP1 (66). On the other hand, it was very recently shown that cigarette smokers who are carriers of BRCA1 or BRCA2 mutations were found to have a lower breast cancer risk than subjects with mutations who never smoked indicating that somehow smoking appears to reduce breast cancer risk in these patients (67). This confuses the significance of published observations in the field and indicates the amount of work which lies ahead in order to clarify our

understanding of the interaction of environmental carcinogens and breast cancer genetic predisposition.

SOMATIC CHROMOSOMAL AND GENETIC ABNORMALITIES IN BREAST CANCER

Cytogenetics of Breast Cancer

Numerous studies have been performed in order to characterize the role of chromosomal abnormalities in breast cancer. However, as is the case with other solid tumors of epithelial origin, it has been difficult to identify any primary cytogenetic changes among the large number of apparently random alterations. This is due to the clonal heterogeneity characteristic of breast cancer as well as to the inherent difficulties in obtaining high-quality metaphases from solid tumors. Nevertheless, the prevalence of several specific chromosomal aberrations have been noted. The most frequent tend to be numerical alterations of whole chromosome copy number including trisomies of chromosomes 7 and 18 and monosomies of 6, 8, 11, 13, 16, 17, 22, and X (68). The most common aberrations in non-metastatic near-diploid tumors are, loss of chromosomes 17 and 19, trisomy of chromosome 7, and overrepresentation of chromosome arms 1q, 3q, and 6p (69). Structural alterations include terminal deletions and unbalanced nonreciprocal translocations, most frequently involving chromosomes 1, 6, and 16q. Breakpoints for structural abnormalities cluster to several chromosomal segments, including 1p22-q11, 3p11, 6p11-13, 7p11-q11, 8p11-q11, 16q, and 19q13 (69). In particular, 16q was shown to participate systematically in

translocations with chromosome 1q and to display frequent deletions. In fact, some investigators have suggested that specific abnormalities affecting chromosome 16q could be considered primary cytogenetic aberrations since they were observed in the absence of other anomalies (70,71).

A recently developed molecular cytogenetic technique called comparative genomic hybridization (CGH), allows for the analysis of chromosome copy number abnormalities involving segments of at least 10 Mb (72). Since CGH involves hybridizing differentially labeled genomic DNA from a tumor and a normal cell population to the same normal metaphase, it circumvents some of the difficulties encountered in conventional karyotyping. Through such analyses, nearly every tumor analyzed revealed increased or decreased DNA sequence copy number (73).

The most common regions of increased copy number in breast cancer as determined by CGH include 1q, 8q, 17q22-24, and 20q13. Regions of decreased DNA copy number were also observed and include 3p, 6q, 8p, 11p, 12q, 13q, 16q, and 17p (74). For some of these regional losses, candidate genes exist that may be the target of deletion in the progression to a malignant phenotype (Table 1). These genes and their corresponding regions will be discussed in more detail in the following sections. Interestingly, when both loss and gain of DNA copy number as determined by CGH were compared with survival data in a series of node negative breast tumors, only copy number losses were significant for recurrence and for overall survival (75). However, as is the case with conventional cytogenetics, CGH studies failed to reveal any characteristic abnormalities that occur in the majority of breast tumors or to identify any abnormalities which could be considered "primary".

Oncogenes and Gene Amplification

In human breast cancer as in other solid tumors, the most common aberration affecting oncogenes appears to be gene amplification. Abundant evidence demonstrates that huge regions of DNA, up to entire chromosome arms, can be amplified as a contiguous unit. The importance of this to breast cancer development is still unclear although it suggests that genes within these regions are overexpressed, due to their high representation. Chromosomal regions overrepresented in tumor cells suggest the presence of activated oncogenes. Proto-oncogenes encode proteins involved in cascade of events leading to growth in response to mitogenic factors. Alteration in the normal function of proto-oncogenes, through mutation or increased expression, can result in a constant growth stimulus and a constitutive mitogenic response. Aberration of a single allele of an oncogene can be sufficient to lead to altered signal and as such is dominant. Current data suggest that of the numerous oncogenes described to date, only a few may have a role in breast tumorigenesis.

Regions Affected by Gene Amplification

Region 17q12 (ERBB2)

In 1987, ERBB2 was demonstrated to be overexpressed and amplified in 20-40% percentage of breast cancers (76,77). Amplification was shown to be consistently accompanied by increases in mRNA and protein levels (76,77). In later studies, increased copy number of the long arm of

chromosome 17 (17q) which contains the ERBB2 gene, demonstrated a 50 to 100 fold amplification in some cases as determined by gene fluorescence in situ hybridization analysis (72).

The fact that ERBB2 is overexpressed in a high percentage of breast cancers implicates its involvement in breast tumorigenesis (78,79). Therefore in recent years the diagnostic and possible treatment value of ERBB2 detection has been extensively studied. Early studies reported a prognostic value of ERBB2 overexpression in node negative breast cancer. However, more recent studies using larger data sets did not support these early observations and question the prognostic role for ERBB2 expression in node positive breast cancer. Expression of ERBB2 may have value in predicting response to specific therapies, additional studies are underway to confirm these observations (reviewed by Ravdin & Chamness 1995). For instance, ERBB2 overexpression has been associated with increased resistance to chemotherapy (82) and estrogen receptor positive patients who overexpress ERBB2 are less likely to respond to hormone therapy (83).

Studies have also shown that activation or overexpression of ERBB2 in transgenic mice results in the genesis of mammary tumors (84). Whereas neutralizing antibodies against ERBB2 lead to tumor regression (85). The possibility of using antibodies against ERBB2 as a means of treating breast cancer is presently under investigation (86,87).

The ERBB2 proto-oncogene is a member of the epidermal growth factor receptor family. All of the family members which include EGFR, ERBB2 (Her-2/neu) ERBB3 and ERBB4, have demonstrated overexpression in breast cancer. In addition, overexpression of ligands for these receptors, such as TGF-alpha, have been associated with neoplastic transformation in transgenic mouse models (88). This family of receptors encodes

transmembrane glycoproteins with tyrosine activity. However, although the family members share high homology their ligand specificity is distinct. It has been suggested distinct biochemical and biological responses of the individual receptors such as ERBB3 and ERBB4 (89). Therefore although these receptors may show similarity in their ability to regulate cell proliferation their mechanism of action is most likely diverse.

Region 8q (c-Myc)

Amplifications at region 8q including the oncogene *c-myc,* a gene known to be overexpressed, either by amplification or regulatory means, in breast cancers (90). c-myc is a member of a small family of related proteins that function as sequence specific transcription factors (91). Activation of the *c-myc, Nmyc and Lmyc* genes has been described in many human cancers (92). In normal cells, *c-myc* expression is rapidly induced following mitogenic stimulation, and its activity is absolutely dependent upon the presence of growth factors (93). The c-myc protein is commonly implicated in mediating the transition of cells from quiescence to proliferation (94). Therefore, c-myc is considered to be a positive regulator of cell growth and its activation is thought to confer a growth advantage upon a tumor cell. Conversely, c-myc has also been demonstrated to induce apoptosis a function more consistent with a negative regulator of cell growth (95,96). A possible explanation for this bifunctional activity lies in the fact that in order for c-myc to act as a promoter of cell proliferation appropriate serum growth factors, stimulating growth via a separate pathway, must be present (92). In the

absence of growth regulators, over expression of c-myc is sending conflicting signals to the nucleus, which in turn initiates programmed cell death (i.e. apoptosis).

The *myc* gene has been shown to be amplified in approximately 25% of breast carcinomas (90). Overexpression of *c-myc* in transgenic mice results in mammary tumors (97), and amplification of *c-myc* has been associated with high grade tumors in humans (98). Of additional interest, *c-myc* expression is modulated by the presence of estrogen in estrogen-responsive cell lines, and constitutively high *c-myc* expression is observed in hormone-dependent lines, probably because of increased stability of the transcript (99).

Region 11q13 (cyclin D)

Chromosome region 11q13 has also been reported to be amplified in 15-20% of breast cancers and is associated with poor prognosis (100). The *cyclin D1* gene, located in this region, is thought to be the target of such amplification. Cyclin D1 is overexpressed in 45% of breast carcinomas, most of which are both estrogen and progesterone receptor positive (101,102). Studies show that transgenic mice homozygous null for *cyclin D1* fail to undergo proliferative changes of the mammary epithelium associated with pregnancy, thereby indicating a role for *cyclin D1* in steroid-induced proliferation of the mammary epithelium (103). Transgenic mice overexpressed *cyclin D1* have been shown to develop mammary carcinomas (104). Analysis of *cyclin D1* expression by mRNA *in situ* hybridization has shown a dramatic increase of *cyclin D1* expression

in 76% of low grade carcinoma *in situ,* further suggesting a role for cyclin D1 in the tumorigenesis of the breast (105).

Mapped within this same region is the *int-2* gene. Transgenic mice strains containing the *int-2* transgene develop multifocal preneoplastic hyperplasia of the mammary gland which can give rise to focal mammary tumors (106). The possibility that an additional gene responsible for breast tumorigenesis within this amplified region is *int-2* is controversial since corresponding increases in mRNA and protein levels for *int-2* rarely correspond to amplification status. Thus, the possibility remains that a yet unknown gene located close to *int-2* might have a biological effect, whether *int-2* itself is merely co-amplified remains to be seen.

Region 20q13 (AIB1)

The gene (AIB1), amplified in breast cancer, was very recently identified as a leading candidate for the amplification of region 20q13 (region described to be amplified in 15-30% of cases) (107). AIB1 was found amplified in all estrogen receptor positive cell lines and it has been identified as a nuclear steroid receptor coactivator (107). AIB1 amplification may contribute to the development of steroid-dependent breast cancers by interacting with the estrogen receptor to enhance the effects of estrogen on tumor cells.

Tumor Suppressors and Loss of Heterozygosity

Knudson, on the basis of statistical analysis of clinical observations, was the first to suggest that retinoblastoma was a cancer caused by two mutational events (108). In the hereditary form of retinoblastoma one mutation is germinal; thus only a single additional somatic mutation is required. This resulted in early onset and a tendency toward bilateral tumorigenesis. In the sporadic form, both mutations are somatic, resulting in a tendency toward unilaterally and late onset. It was later suggested that these two mutational events could occur within separate alleles of a regulatory gene (109). Supporting this, cytogenetic analysis of retinoblastoma revealed characteristic deletions of the long arm of chromosome 13. Subsequent analysis of the same chromosome region led to the cloning of *RB1* and identification of aberrant transcripts encoded from the remaining allele (110). As a consequence of these studies, a precedent emerged where inactivation of one allele of a tumor suppressor is accomplished by mutation, leading to the eventual deletion of the remaining normal allele through chromosomal aberrations and thus loss of heterozygosity (LOH) is thereby observed in the suppressor locus. This precedent is now considered the convention for tumor suppressor gene inactivation and similar observations have been made for several other putative tumor suppressor genes (e.g., *APC*, *DCC*, *VHL*, *TP53*; reviewed by (111). Therefore, LOH is considered indirect evidence for the existence of a suppressor gene within the affected chromosomal region.

Breast Cancer Allelotype

The chromosomal mechanisms by which loss of heterozygosity occurs tend to involve large segments of DNA, thus it is possible to utilize adjacent genes or known noncoding sequences as markers to identify deleted regions harboring putative suppressor genes whose loss may be important in the genesis or progression of a tumor. One such genetic marker is the naturally occurring simple sequence length polymorphisms (SSLPs). SSLPs consist mainly of dinucleotide repeats, primarily $(CA)_n$, which are repeated in tandem at variable number interspersed throughout the genome (112). These polymorphic microsatellites have a mean heterozygosity of 70% and recent mapping efforts reported an average spacing of 199 Kb (113). Through known linkage maps and comparison to physical maps, it is possible to select highly polymorphic microsatellites at any position within the genome. Further, through PCR amplification of these microsatellites and comparison with normal DNA from the same patient, it is possible to generate a comprehensive map of allelic imbalances and losses (allelotype) occurring in a neoplasm.

Numerous studies have analyzed the breast cancer allelotype, and numerous regions of allelic imbalance have been described using microsatellites as well as the older restriction fragment length polymorphism analysis. Deville and Cornelisse, reviewed data from more than 30 studies revealing a consensus of imbalances affecting 12 chromosome arms at a frequency of more than 25% (Table 1). Chromosome arms 1p, 1q, 3p, 6q, 8p, 11p, 13q, 17q, 18q, and 22q were affected at a frequency of 25-40%, whereas chromosome arms 16q and 17p were affected in more than 50% of tumors (68). In addition, chromosome arm

9p, has recently been reported to be affected by allelic imbalances and losses in numerous breast carcinomas (114). In general, the loss of genetic material in many of these regions has been corroborated by either CGH or classic cytogenetic data (68,115). Some of these regions are known to harbor tumor suppressive genes whose loss has been demonstrated through a variety of techniques, including Southern blot analysis and FISH using gene-specific single-copy probes (111).

Although there is overwhelming evidence that these genetic losses occur, inherent difficulties exist in determining the relevance of such losses to breast carcinogenesis. In most cases, the tumors analyzed were of the invasive type and/or advanced stages of progression, leading to the question of whether these losses are causative factors of tumorigenesis or consequences of the general genomic instability inherent to tumors. It is possible that certain losses may be selected for in the progression or clonal evolution of a tumor to a more advanced type but not strictly necessary for the genesis of the tumor. Some of these questions could be addressed in part through comparative allelotyping of both noninvasive and invasive tumors.

The relative timing and frequency of allelic losses of commonly affected regions in breast cancer was estimated by comparing the allelotype of preinvasive ductal carcinomas (DCIS) and invasive carcinomas (81). The allelotypic analysis of DCIS samples revealed that chromosomal regions 3p, 3q, 6p, 11p, 16p, 18p, 18q, and 22q were not affected by a high frequency of loss, on the other hand analyses of these same regions of invasive tumors showed them to be affected in 10-40% of cases (81) (Table 1). These findings are in agreement with those of Radford et. al. who examined 61 DCIS samples (116). Since allelic losses

affecting these regions were not frequently observed at the noninvasive (DCIS) stage it can be concluded that alterations of these regions are late events in breast cancer progression. More importantly, allelic imbalances observed on chromosome arms 7p, 7q, 16q, 17p, and 17q (81), as well as 9p as reported by others (117), appear to be early abnormalities because they occur frequently in DCIS (Table 1).

Targets of Allelic Loss

Chromosome Region 17p13

The short arm of chromosome 17, is subject to allelic loss in more than 50% of invasive ductal carcinomas, and approximately 30% of noninvasive ductal carcinomas (81,116,118). This high frequency of allelic loss suggested that a tumor suppressor of relevance to breast tumorigenesis resides in this region. Indeed, tumor suppressor *p53* maps to chromosome band 17p13 and is known to harbor somatic mutation in 25-45% of primary breast carcinomas (49). Recently p53 mutations were identified in mammary ductal carcinoma *in situ* but not in epithelial hyperplasia (119). It has been suggested that p53 mutation analysis may serve as a marker for identifying preinvasive lesions at increased risk of developing invasive carcinoma.

We have already discussed the relevance of germinal *p53* mutations as the cancer predisposing alteration in the Li-Fraumeni syndrome (47,48). In tumors from patients with Li-Fraumeni syndrome, loss of the wild-type allele is observed in conjunction with retention of the mutant *p53* allele. Functional studies of cells with mutant *p53* indicate a change

of phenotypes, including cellular immortalization, loss of growth suppression, and fourfold increase in protein half-life which leads to *p53* accumulation. Accumulation of *p53* protein, observed by immunohistochemical analysis in roughly 30-50% of sporadic breast carcinomas, was proposed to be an indicator of higher risk of recurrence in patients with tumors positive for *p53* expression (reviewed by (120). It appears that *p53* inactivation through mutation and LOH is intrinsically linked to the development of subsequent further genomic instability as suggested by *in vitro* findings, as discussed in a separate chapter, and as demonstrated in experimental models of mammary cancer (121).

Although *p53* is most likely the driving force for allelic loss on 17p, some reports indicate that there may exist another distinct locus that may be a target of allelic loss. In an analysis of 141 breast tumors, Cornelis et al. observed a strong association between *p53* mutation and allelic loss of the *p53* locus (122). However, in cases where *p53* mutation was not observed, allelic loss of distal region 17p13.3 was always observed, sometimes without p53 allele loss. Similar findings of distal deletion of 17p were also observed in DCIS (116). While these findings support the existence of a second gene as target of allelic loss, further studies are needed to address this issue.

Chromosome Region 17q21-22

The long arm of chromosome 17, also frequently affected by allelic imbalance in both familial and sporadic breast cancers, has recently been subjected to extensive analysis because 17q has been linked to familial breast cancer (123). As a result, the *BRCA1* gene was isolated by

positional cloning as discussed in preceding sections (29). However, when sporadic breast tumors with allelic loss of 17q were examined for *BRCA1* coding sequence alterations, only about 10% of those with LOH revealed any change of sequence, and those mutations were found to be germinal (124).

Another known putative suppressor gene localized in this region, nm23 or *NME1*, has been shown to undergo allelic loss in as much as 60% of breast carcinomas (125). However, analysis of *NME1* has not revealed evidence of mutations (126). An additional possible explanation for allele loss in 17q is the existence of a yet-unidentified gene within this region as the target for LOH (122).

Chromosome Region 13q14

Loss of the *RB1* region 13q14 has been reported for numerous neoplasms including small cell lung carcinoma, bladder carcinoma, osteosarcoma, and breast carcinoma (reviewed by (111). These losses appear to be relatively early losses in some tumors since 15-20% of tumors at the DCIS stage reveal allelic loss of 13q (81,116). However, when allelic loss and expression are examined in the same breast tumors, no correlation between the two is observed, suggesting that Rb inactivation is not acquired by allelic loss and that another gene may be the target of such inactivation (127). As discussed in a previous section, a second breast cancer susceptibility gene, *BRCA2*, was mapped to chromosome 13q12-13 (43). This suggested that the *BRCA2* gene may be involved in sporadic breast cancer as well. However, similar to the findings with *BRCA1* on 17q, when sporadic breast tumors were analyzed

for mutation of BRCA2, mutations were infrequent, indicating that *BRCA2* is not the gene being targeted by loss (128-130). Brush-1 is another gene that has been mapped to 13q12-13, proximal to *RB1*. Analysis of Brush-1 expression showed it to be low to absent in 6 of 13 breast cancer lines and decreased in four of four tumors showing LOH of 13q12-13 (131). However, no sequence analysis has yet been reported, and the question of whether decreased expression of Brush-1 results from allelic loss involving large regions of another gene has yet to be addressed.

Chromosome Region 16q

Chromosome 16q has been suggested as a site for the occurrence of primary cytogenetic structural abnormalities in the development of breast cancer (70,71). In particular the long arm of chromosome 16 was shown to systematically participate in nonrandom translocations with chromosome 1. Breast cancer allelotypic studies have also shown the common occurrence of allelic losses affecting the long arm of chromosome 16 (132-134). Several studies have reported the occurrence of frequent allelic losses affecting chromosome 16q in DCIS (81,116,133).

It has been suggested that more than one putative tumor suppressor resides in the chromosome region 16q. At least two regions of chromosome 16q have consistently been reported to show LOH: 16q21 and 16q24.2-qter (132-134). In most recent studies, high-resolution allelotyping of chromosome 16 in DCIS lesions have identified three distinct regions with a very high incidence (about 70% or more) of allelic losses (41). Two of the three regions agree with previously described areas: 16q21 at locus D16S400 and 16q24.2 at locus D16S402 (41).

However, the region with the highest incidence of LOH observed, lies within 16q23.3-q24.1 close to marker D16S518 (41).

E-cadherin (CDH1), a cell adhesion molecule implicated as an invasion suppressor protein, is one possible candidate target of the LOH at chromosome 16q21. Interestingly, this gene was demonstrated to be mutated at a high frequency in invasive lobular carcinomas of the breast. The lack of E-cadherin expression is believed to be the cause behind the infiltrative growth pattern characteristic of lobular carcinomas (135). However, the more common, invasive ductal carcinomas do not show high incidence of E-cadherin mutations. In addition to mutation E-cadherin may be inactivated by CpG methylation within the gene's promoter region (136,137). Expression of a second cadherin gene, H-cadherin, map to region 16q24 (138) was reported to be absent or reduced in several breast cancer cell lines. Further studies are necessary to identify additional possible targets for the common allelic losses observed to affect this autosome in breast cancer.

Chromosomal Region 9p

Chromosomal region 9p21, as previously discussed, has been shown to be affected by allelic loss or imbalance in more than 58% of invasive ductal carcinomas and 30% of DCIS, suggesting it may be involved in breast tumorigenesis (114,117). Previously, the p16 tumor suppressor gene was identified within this region by positional cloning and shown to be affected by homozygous deletions in 60% of breast carcinoma lines (139). However, when primary breast tumors were analyzed for mutation of the *CDKN2* coding region, few mutations were found (114). More recent

analysis, including FISH determination of gene copy number, methylation of the 5' region, and analysis of expression, indicate that *p16* appears to be a target of abnormalities in approximately 40% of breast tumors (140). These observations substantiate a role for *p16* inactivation in the tumorigenesis of the breast and as a target of 9p allelic loss. Interestingly, however some breast tumors show overexpression of p16 indicating that involvement of this gene as well as that of $p14^{ARF}$ (homolog of mouse $p19^{ARF}$), encoded at the same locus in an alternative reading frame is more complex than previously thought, as will be discussed in the following section.

CELL CYCLE IN BREAST CANCER

Cell replication in eukaryotes proceeds through an orderly cascade of events manifested as the cell cycle. The machinery responsible for such progress includes a hierarchy of proteins and complexes each exerting an effect on the next. At the top of this hierarchy are the cyclin subunits, whose expression and stability oscillate in a phase-dependent manner. The expression of certain cyclin genes can be upregulated by different mitogenic stimuli, for example, the upregulation of cyclin D1 by estrogen (141). Each of these cyclins can associate in a specific manner with corresponding cyclin-dependent kinases (CDKs). Cyclins are in competition with CDK inhibitors, which have the ability to displace the cyclin and form an inactive complex with the CDKs. When CDKs are active, they phosphorylate, and hence inactivate, other proteins with transcription-repressing activity (Reviewed by Sherr, 1996) (Figure 5).

Of the restriction points, G1 to S is best characterized in breast cancer. Key players in early G1 and after the passage of cells from G0 to G1, include cyclins D1-D3, CDKs 4 and 6, the specific inhibitors of these CDKs, p15, p16, p18 and p19 and the substrates of CDKs, Rb and Rb-like proteins. Later in G1 and fueled by E2F1 transcriptional activation, cyclin E and its partner CDK2, become important players in the G1-S transition. Collectively, these proteins are known elements responsible for regulating progression trough G1 and as a consequence loss of function or abnormalities in the expression of an individual protein can lead to cell cycle dysregulation and altered cell proliferation.

An additional family of CDK inhibitory proteins also exists which includes, $p21^{cip1}$, $p27^{kip1}$ and $p57^{kip2}$. Of the proteins mentioned, the Rb protein, cyclin D1, cyclin E, p16 and p27 have all been observed to be affected in breast carcinogenesis. As previously mentioned, cyclin D1 has been shown to be both amplified in 10-20% of breast tumors and overexpressed in the majority of breast tumors (101,102,105). Cyclin D1 competes with *p16* for heterodimerization with the CDKs. When cyclin D1 is more abundant than p16, it binds to and activates CDK4 and CDK6 (Figure 5). Recently cyclin D1 mRNA and estrogen receptor expression were found to be positively correlated in primary breast cancer (144). There is no conclusive evidence however demonstrating that estrogen receptor directly up regulates cyclin D1 transcription.

Recent studies have suggested that overexpression of cyclin E, which is often found in breast tumors, is functionally redundant to cyclin D (145). In cells overexpressing both cyclin E and p16, cyclin E can functionally replace cyclin D providing tumor cells with a growth advantage (145). They do this by activating CDK's which in turn

phosphorylate *Rb*, releasing E2F and initiating gene transcription, leading to cell cycle progression and a self-perpetuating positive regulatory loop (Figure 5). Interestingly, it was recently reported a bad prognosis and very high mortality rate in women with breast cancers showing high cyclin E expression concomitant with low expression of the CDK inhibitor $p27^{kip1}$ (146).

Inactivation of *Rb* itself has been described in breast cancer as a means of enhancing cell cycle progression. Although, when multiple modes of inactivation are accounted for, *Rb* is inactivated in less then 20% of breast cancers (127,147). In the vast majority of tumor lines there is an inverse relationship between *Rb* and *p16* expression (148,149). (i.e., breast tumor cell lines which retain *Rb* expression have no expression of *p16*. Whereas, cell lines retaining *p16* expression often lack expression of *Rb*.) When primary breast tumors were analyzed for *p16* expression, approximately 50% showed loss or reduced expression (140). This loss of expression may be due to homozygous deletion, methylation or in rare instances mutations (140).

The CDK inhibitor *p21* CIP1 as indicated, is a universal inhibitor of CDKs, inducing cell cycle arrest at both the G1/S and G2/M restriction points (150). Inhibition of DNA replication occurs when *p21* complexes with the proliferating cell nuclear antigen, PCNA (151). Because *p21* gene transcription is regulated by *p53*, it has been suggested that *p53*-dependent cell cycle arrest is mediated by *p21*. Indeed, *p21* nullizygous mice fibroblasts fail to undergo G1 arrest when p53 is activated following DNA damage, although apoptosis is unaffected and occurs when p53 is activated in these same cells (152).

As previously mentioned, positive detection of the p53 protein accumulation has been shown to be associated with p53 mutations and a higher risk of breast cancer recurrence (reviewed by Ozbun and Butel, 1995). Therefore, p53 inactivation appears to be a critical event in the tumorigenesis of the breast. This also suggests that an additional consequence of p53 inactivation would be abrogation of cell cycle arrest through loss of transcriptional activation of p21.

Interestingly, very recently it was demonstrated an important regulatory link between both the P53 and Rb pathways. At the center of this link is the recently described $p14^{ARF}$ (previously called $p19^{ARF}$ because of the mouse homolog). As mentioned in a preceding section, this gene is encoded at the INK4a locus in chromosome 9p21, as an alternative reading frame of the cyclin dependent kinase inhibitor $p16^{ink4a}$. It was demonstrated that the putative tumor suppressor ARF gene physically interacts with MDM2 and as a consequence basically blocks MDM2-induced p53 degradation and transcriptional inactivation (153,154). Thus, this interaction leads to increase p53 stability and accumulation (Figure 5). Further strengthening the connection between the p53 and Rb pathways, it was very recently reported that ARF is transcriptionally upregulated by E2F1 (155). This allowed to speculate that perhaps abnormal cell proliferation, which results in E2F1 increase (Figure 5), in turn would result in increase of ARF which would lead to cell arrest or apoptosis via p53. This would not happen if an additional abnormality takes place such as p53 mutation or ARF inactivation (155). In studies from our laboratory we have demonstrated that both ARF and p16 expression levels are highly variable in breast cancer (140). We observed subsets of tumors that lack expression of both genes (p16 and ARF) due to common inactivation events

such as homozygous deletion. On the other hand we have also observed numerous tumors that dramatically overexpressed ARF. We are currently addressing the p53 status in the same tumors.

APOPTOSIS AND BREAST CANCER

Closely linked to the deregulation of the cell cycle are the molecular pathways leading to programmed cell death (i.e. apoptosis). When DNA is altered during replication, control checkpoints stop the mitotic cycle in order to repair the damage. If the DNA cannot be repaired, apoptosis is induced. At first much of cancer research focused on uncontrolled cell proliferation, now researchers are aware of the important role cell death has in maintaining homeostasis.

Much remains to be done in terms of understanding the sequence of events that occur during apoptosis, which of them are essential of the process and their role in carcinogenesis. As already mentioned, p53 plays an important role in directing cells into apoptosis when DNA damage occurs. Other key players include the family of Bcl proteins and ICE (interleukin 1β converting enzyme).

BcL2 is a potent repressor of apoptosis conferring survival advantage to cells expressing it. On the other hand, Bax, another Bcl2 family member, is capable of countering the death repressor activity of Bcl2. Bax has been found to exist as a homodimer and is also capable of forming heterodimers with Bcl2 *in vivo* (156). Therefore the ratio of Bax to Bcl2 in a given cell may dictate whether the cell survives upon receiving apoptotic stimuli. This type of homeostatic control by the ratio of homo and heterodymers can be expanded to include several members of

the Bcl2 family. Some Bcl2 members acts as promoters of apoptosis (i.e. Bax) while other members inhibit apoptosis (i.e. Bcl2). Although not a member of the Bc12 family, cMyc has been shown to cooperate with Bcl2 to achieve inmortalization of tumor cells (92). Bcl2 protein expression is highly variable in breast cancer and it was suggested that high expression associated with favorable clinicopathological features (157,158). Recently a study analyzed Bcl2, Bax, Bcl-x and Bak expression and loss of apoptosis in small, non-metastatic breast carcinomas (159). Bcl-2 expression but not Bclx expression was associated with loss of apoptosis. Expression of Bax and Bak was found significantly associated with increased apoptosis in the breast carcinomas. These large gene families form complex set of interactions which may balance the scale either towards or away from apoptosis. It is still premature however to determine whether alterations in pathways of apoptosis play a relevant role in breast carcinogenesis.

ESTROGEN AND BREAST CANCER

The role of estrogen as an important factor in the etiology and progression of human breast cancer has been well documented. It was already observed 100 years ago that ovariectomy could lead to breast cancer regression in premenopausal patients (160). The extent of exposure to ovulatory cycles is one of the most important endogenous causes associated with a higher risk for development of sporadic breast cancer (4). However, while the association of estrogen in the development of breast cancer is well established, the fundamental mechanism(s) by

which this hormone modulates cell growth and tumor development are not yet clear.

It is known from *in vitro* and *in vivo* studies that estrogen's mechanism of action is via its ability to bind the estrogen receptor (ER) which in turn binds specific enhancer regions on the DNA and regulates gene transcription (161). The interaction of estrogen with its receptor and the recruitment of accessory cofactor proteins to bind DNA and activate gene transcription has been the focus of intense recent research (162). However we understand very little downstream from these events. Important questions still remain such as; What are the main gene targets upon which estrogen acts to exert a growth response? What is the chronology of such events?

Estrogen has been shown to increase the pool of cells synthesizing DNA by recruiting non-cycling cells into the cell cycle and by reducing the length of the G1 phase (163). The ability of estrogen to regulate the transcription of c-Myc and c-Fos is believed to be, in part, responsible for estrogen's stimulatory effects on the cell cycle (164). Entry into S phase was found to be preceded by increased activity of both Cdk4 and cyclin E-Cdk2 and hyperphosphorylation of pRB, all within the first 3-6 hours of estradiol treatment (165). The increase in Cdk4 activity was accompanied by increases in cyclin D1 mRNA and protein, indicating that an initiating event in the activation of Cdk4 was increased cyclin D1 gene expression. In addition to cyclin D1's ability to activate cdk4, cyclin D1 has recently been shown to directly enhance transcription of estrogen receptor related genes (166). Cyclin D1 does this without binding cdk4 and in the absence of estrogen, thereby identifying an additional role for cyclin D1 in promoting cell growth.

However the effects of estrogen on cyclin D1 expression may not be a direct effect of the estrogen receptor on the cyclin D1 gene promoter. The ability of protein synthesis inhibitors to abolish cyclin D mRNA induction by estrogen, suggests intermediary proteins could be involved (165). Clearly our understanding of how estrogen exerts its effects on breast tissue requires further analysis.

NEW TECHNOLOGIES AND FUTURE DIRECTIONS

It is estimated that within the next few years we will have compiled gene sequence information for the entire human genome. However if we are to use the tremendous amount of information gained to improve the treatment of breast cancer patients, it is imperative that we bridge the gap between genes and their relationship to a particular physiopathological outcome. To date the majority of molecular biology research has focused on abnormalities of the genome such as mutation, gene amplification and loss of heterozygosity as discussed in preceding sections. Identifying defects in the genome associated with breast cancer is the first level of genomic complexity. The next level of complexity is characterizing the changes in gene expression as a cell progresses from normal to malignant.

Present day advances in gene expression technology are allowing researchers to study this next level of genomic complexity by defining global changes is gene expression. Technologies such as SAGE (Serial Analysis of Gene Expression) and Microarray technologies are at the cutting edge of cancer research. Ultimately, the ability to understand the detailed mechanisms of tumor progression, from the very early stages of

carcinogenesis through metastasis will allow researchers to identify key components and interactions of the malignant pathway. Recently, the feasibility of SAGE was demonstrated by analyzing more than 300,000 transcripts derived from at least 45,000 different genes in both normal and neoplastic cells (167).

The knowledge found by defining global and specific alterations in the transcription of premalignant and malignant cells, would allow researchers to concentrate on gene targets that will better serve as diagnostic and prognostic tools. Ultimately it would be ideal to achieve a very precise matching of treatment to individual tumors profiles. A logical additional consequence will be the designing of more rationale therapeutic approaches.

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Figure Legend

Figure 1. Diagrammatic depiction of the multiple endogenous and exogenous factors which contribute to breast cancer risk. Ultimately, it is the combination of multiple factors along with the unique genetic composition of each individual woman that plays a decisive role in defining the risk for tumor development. This etiologic complexity is also responsible for the characteristic heterogeneity of breast cancer.

Figure 2. A diagrammatic representation of the normal breast structure and histology (modified from Tavassoli 1992).

Figure 3. A graphic depiction of the 'sporadic' breast cancer incidence as compared to hereditary breast cancer incidence.

Figure 4. Schematic putative model of breast cancer histopathological progression and corresponding estimated breast cancer risk based on studies by Page and Dupont (Page DL, Dupont WD, 1992). A woman with PDWA (proliferative disease without atypia) has a 1.5 to 2 times greater risk for developing breast cancer as compared to the general population. Whereas, a women with atypical hyperplasia (AH) has 4-5 times greater risk. Women with DCIS (ductal carcinoma in situ) and LCIS (lobular carcinoma in situ) are at a much higher risk of progressing to invasive cancer. LNG is low nuclear grade and HNG is high nuclear grade

Figure 5. Schematic representation of cell cycle's G1/S key restriction point controls many of which are known to be altered in breast cancer (see text).

activity of ts72*src*, ii) the activity of *jun* N-terminal kinase (JNK), iii) the level of MAP kinase (ERK2) protein, or iv) the induction of collagenases and gelatinases associated with tumor invasiveness. However, ERK2 activity was induced 5- to 10-fold by SSeCKS in the presence of active *src*. SSeCKS reversed v-*src*'s ability to decrease the formation of vinculin-associated adhesion plaques, actin-based stress fibers and filopodia-like structures. These data suggest a tumor suppressive role for SSeCKS via the control of cytoskeletal architecture and cell signaling.

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#3804 Extended life of normal HMEC cultures through loss of p16^{INK4a} expression. Brenner, A.J., Stampfer, M.R., and Aldaz, C.M. *Department of Carcinogenesis, University of Texas M.D. Anderson Cancer Center, Science Park-Research Division, Smithville, TX 78957, Lawrence Berkeley Laboratory, Berkeley, CA 94720*

Numerous reports have implicated the p16^{INK4a} tumor suppressor as a candidate senescence gene. Previously, we reported loss of p16 in 4 of 4 immortal breast epithelial cultures derived from normal epithelium. In order to address the status of p16 in normal human mammary epithelial cells (HMEC), we have begun analyses of p16 expression in both primary and established HMEC cultures. Primary HMEC cultures initially display low levels of p16 but increase p16 with increased population doubling level until senescence. It has been observed that when cells are grown beyond their typical replicative span in medium MCDB 170, the cells appear to undergo a selection process in which subpopulations of actively growing cells emerge. Interestingly, we observed that these post selection cells fail to express p16. This loss of expression was observed only when cells were grown in MCDB 170 plus serum free supplements, a medium previously shown to confer an extended proliferative capacity to HMEC cultures. This data would suggest a role for the p16^{INK4a} tumor suppressor in the control of replicative senescence. We are currently investigating the mode of p16 transcript loss and involvement of p53, as well as p16 and p53 cooperativity in immortalization.

#3805 Downregulation of *p16/CDKN2* tumor suppressor promoter activity by methylation at critical CpG sites. Gonzalgo, M.L., Hayashida, T., Pao, M.M., Bender, C.M., Tsai, Y.C., Gonzales, F.A., and Jones, P.A. *Department of Biochemistry & Molecular Biology, USC/Norris Comprehensive Cancer Center, University of Southern California School of Medicine, Los Angeles, CA 90033*

Methylation of CpG sites in the control regions of tumor suppressor genes may be an important mechanism for their heritable, yet reversible, transcriptional inactivation. These changes in methylation may impair the proper expression and/or function of cell-cycle regulatory genes and thus confer a selective growth advantage to affected cells. We used bisulfite genomic sequencing to find key CpG dinucleotides associated with downregulation of putative transcriptional start sites in the *p16/CDKN2* promoter, in a series of subclones of a bladder cancer cell line in which the hypermethylated gene had been reactivated by transient treatment with 5–Aza-2′-deoxycytidine. The importance of four CCGG sites was confirmed by *in vitro* methylation experiments which produced a 4-fold reduction in CAT reporter gene expression compared to an unmethylated CAT expression vector containing the *p16* promoter. These experiments yield valuable insight into the mechanisms of tumor suppressor gene inactivation by the epigenetic effects of DNA methylation and also suggest that methylation inhibitors such as 5-Aza-2′–deoxycytidine may be useful for reactivating dormant growth regulatory genes that have been silenced by DNA methylation.

#3806 Anti-invasion effect of p16 in malignant glioma. Rao, J., Chintala, S., Venkaiah, B., Gomez-Manzano, C., Fueyo, J., and Kyritsis, A. *Department of Neurosurgery, U.T.M.D. Anderson Cancer Center, 1515 Holcombe Blvd., Houston, TX 77030, USA*

The diffuse invasion of the brain parenchyma by malignant astrocytomas is one of the most important barriers to successful therapy. Better understanding of the regulators of the proliferation and invasion of these tumors may help to improve current therapy. To address the issue of what is the potential involvement of p16 gene in glioma invasion we used a recombinant replication deficient adenovirus to infect and transduce high levels of p16 protein to null-p16 human glioma cells. Assays for invasion using Matrigel-coated transwell inserts showed that the SNB19 and U-251 MG cells infected with p16 had significant reduction of invasion. The p16-mediated anti-invasion effect was further tested in co-culture experiments using fetal rat brain aggregates and tumor spheroids. SNB19 cells expressing exogenous p16 showed decrease in the ability to invade into fetal rat brain aggregates during a 72 h time period compared to parental SNB19 and vector infected cells, as assessed by Confocal Laser Scanning Microscopy. Moreover, the expression of 72-KDa matrix metalloprotease (MMP-2) in p16-infected SNB19 cells was significantly reduced compared to mock and vector-infected cells. The present results indicate that inactivation of p16 may facilitate glioma invasiveness. In addition, our data suggest a novel function of p16 and indicate that restoration of wild-type p16 activity may have therapeutic utility for glioma.

#3807 p16/INK4A/CDKN2, p53 and pRB did not predict chemosensitivity of the human lung cancer cell lines to anti-cancer agents. Chikamori, K., Matsushita, A., Tabata, M., Kohara, H., Aoe, K., Kiura, K., Ueoka, H., and Harada, M. *Department of Medicine II, Okayama University Medical School, Japan*

The CDKN2 tumor suppressor gene encodes p16/INK4A, an inhibitor of Cdk4 mediated phosphorylation of pRB at the G1-S cell cycle. p16/INK4A is frequently inactivated in non-small cell lung cancer (NSCLC), but rarely in small cell lung cancer(SCLC). As NSCLC is generally more resistant to anti-cancer agents than SCLC, p16/INK4A might control tumor sensitivity to anti-cancer agents. To elucidate relationships of p16/INK4A/CDKN2, p53, and pRB to chemosensitivity, we examined homozygous deletion or mutation of these genes by PCR-SSCP, and expression by RT-PCR and Western blotting among 33 human lung cancer cell lines(19 NSCLC and 14 SCLC). Cytotoxicity of 22 anti-cancer agents were tested by MTT assay. Homozygous deletion or mutation of CDKN2 gene was disclosed in 7 (37%) of 19 NSCLC and 2 (14%) of 14 SCLC cell lines. Loss of expression of p16/INK4A was observed in 12 (63%) of NSCLC and 3 (21%) of SCLC cell lines. An expression level of p16/1NK4A was inversely related to that of pRB but not of p53. Although low sensitivity to paclitaxel and high sensitivity to cytarabine were shown in the cell lines with abnormal p16/INK4A/CDKN2, the difference was not significant. No correlation was observed between p53 and chemosensitivity of the cell lines. These observations indicate that abnormality of an individual tumor suppressor gene did not predict chemosensitivity of the human lung cancer cell lines to anticancer agents.

#3808 Absence of p21 expression in testicular tissue. DeAngelis, T., Jennings, S.B., Brass, A.L., Garcia, F.U., and Rukstalis, D.B. *Allegheny University of the Health Sciences, Department of Urology, Philadelphia, PA 19129*

The relation between the expression of p53 and its downstream effector gene, p21, in germ cell tumors of the testis was analysed in this study, since the p21 encoding gene, WAF1, is located on chromosome 6, which is deleted in 21% of testis cancers. Fourteen primary and metastatic tumor samples were stained with p21 antibody and a panel of p53 antibodies (clone DO-1, DO-7, 240, 1801 and 1620). 4 testicular cancer cell lines (Tera-1, Tera-2, HT-E and HT-H) were tested for the expression of p53 and p21 by Northern and Western blots. Positive staining for p53 (clone DO-7 and DO-1) was seen in spermatogonia and spermatocytes in normal seminiferous tubules, and in 93% of the tumors, including seminomas, cellular elements of mixed germ cell tumors and cells in intratubular germ cell neoplasia. Clone 1801 staining was specific for spermatogonia and spermatocytes, while staining with clone 1620 was abundant during later stages of differentiation. Tumor showed strong positive staining with clone 1620 and negative staining with clone 240, the latter being sporadically restricted to spermatogonia and spermatocytes. Immunostaining for p21 was negative in normal testicular tissues and positive in only 14% of the tumor samples. p21 expression was negative in the cell lines by Northern and Western blots. p53 was undetectable by Northern blot. Since mutations in the p53 gene in testis cancer have not been found the reported results indicate an increased expression of the wild-type p53 protein. However, this expression is uncoupled from the induction of p21, which suggests that different conformations of p53 might interfere with the established p53-p21 pathway.

#3809 Mechanisms of p21 K-ras activation in colon carcinoma. Paty, P.B., Wojciechowicz, D.C., Picon, A.I., and Debnath, G. *Colorectal Service, Dept. of Surgery, Memorial Sloan-Kettering Cancer Center, New York, NY 10021*

Point mutations in codons 12, 13, or 61 produce intrinisic activation of p21ras proteins and are the most widely studied mechanism of ras activation in human cancer. To evaluate whether variations in ras oncogene expression are important in colon cancer, we have studied p21 K-ras, N-ras, and H-ras expression in eleven human colon carcinoma cell lines by western blotting with specific antibodies. p21 K-ras was found to be the predominant form of p21ras expressed in all cell lines. Furthermore, p21-K-ras was relatively overexpressed (2–6 fold) in all eight cell lines containing a K-ras point mutation. In contrast, p21-N-ras and p21-H-ras were expressed at low levels with little variation among cell lines. In addition, the proportion of cellular p21ras found in the activated, GTP-bound state ranged from 3% to 56% and was found to depend upon both K-ras point mutation and p21-K-ras overexpression. We conclude that ras activation varies widely in colon carcinoma and in direct proportion to K-ras oncogene expression.

#3810 p53 gene aberrations in non-small cell lung carcinomas from a smoking population. Liloglou, T., Ross, H.L., Prime, W., Donnelly, R.J., Spandidos, D.A., Gosney, J.R., and Field, J.K. *Molecular Genetics and Oncology Group, Department of Clinical Dental Sciences, The University of Liverpool, L69 3BX, UK [T.L., H.L.R., W.P., J.K.F.], Department of Pathology, The University of Liverpool, UK [J.R.G.], Cardiothoracic Centre, Broadgreen, Liverpool, UK [R.J.D.], National Hellenic Research Foundation, Athens, Greece [D.A.S.]*

Lung cancer development is strongly related to environmental agents and smoking appears to be responsible for the majority of the cases. We examined 46 NSCLC tumours for the presence of p53 mutations in exons 4–9, positive p53 immuno-staining and loss of heterozygosity in the TP53 locus. p53 mutations were detected in 13 tumour samples (28.3%) while overexpression of the p53 protein was found in 30 of 45 (67%) samples. Allelic loss was found in 9 of 38 (23.6%) informative cases. All but three individuals in this study group smoked. In contrast with previous reports, we found a prevalence of GC→AT transitions

MOLECULAR BIOLOGY 18: Deregulation of Cell Cycle Controls in Cancer

#2999 Overexpression of p16 tumor suppressor and cyclin dependent kinase-4 (CDK4) in a subset of human soft tissue sarcoma. Yao, J., Pollock, R.E., Lang, A., Tan, M., Pisters, P.W., Goodrich D., Yu, D. *Surgical Oncology Department, University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Blvd., Houston, TX 77030.*

The $p16^{INK4a}$/MTS1 (p16) gene encodes a specific inhibitor of cyclin-dependent kinase-4 (CDK4) and CDK6. The p16 gene is frequently mutated or deleted in many types of cancer cell lines as well as in certain types of primary tumors. p16 knockout mice are viable but predisposed to sarcoma and B cell lymphoma. To investigate the role of p16 in human soft tissue sarcoma tumor progression, we examined the p16 gene by Southern blot analysis and PCR sequencing in 30 pairs of primary soft tissue sarcomas and autologous normal tissue. Only one tumor sample showed possible rearrangement of the p16 gene. In contrast, western blot analysis of the p16 protein in 20 pairs of samples showed elevated p16 expression in 40% of the tumors when compared with the normal controls. On the other hand, the p16 target protein CDK4 was found to be overexpressed in about 60% of the tumors. In most of the cases CDK4 overexpression accompanied elevated p16 and/or RB levels. Interestingly, a 29kDa protein which can be recognized by a p16 antibody is highly expressed in more than 60% of the tumors. Whether the elevation of p16 is a compensatory result caused by CDK4 overexpression is currently under investigation.

#3000 Analysis of $p16^{Ink4a}$ expression in human breast cancer, correlation with prognostic indicators. Aldaz, C.M., Brenner, A.J., and Haraway, E. *Univ. of Texas. M.D. Anderson Cancer Center, Smithville, TX 78957.*

We and others have previously demonstrated that expression of $p16^{Ink4a}$ (CDKN2), a cyclin dependent kinase inhibitor, is highly variable in human breast cancer. More than 45% of breast tumors show lack or very reduced expression of this transcript. On the other hand a considerable number of breast tumors are found to overexpress p16. To help elucidate the significance of the variable expression of this gene and to investigate its potential role as a prognostic tool, we investigated the association of p16 expression levels with other parameters of prognostic significance in breast cancer. So far we have analyzed a set of 41 human breast cancer samples and observed that low p16 levels are significantly associated with tumors that are positive for detection of Cyclin D1 by immunohistochemistry. On the other hand tumors overexpressing p16 were negative for Cyclin D1 expression (p value = 0.03). Interestingly these tumors with high p16 expression were also negative for progesterone receptor expression, (p value = 0.005). These same tumors had also a tendency to be estrogen receptor negative and lymph node positive for metastases and with a high S phase fraction. Rb inactivation appears not to be the reason for high p16 expression since these breast tumors were found to be positive for Rb expression. This information suggests that tumors with p16 overexpression and Cyclin D1 negative may represent a more advance stage of progression perhaps unresponsive to the growth suppressive effects of p16 expression. On the other hand tumors with low p16 expression levels and positive for Cyclin D1 expression may represent an earlier stage of development. Analysis of p16 expression levels may be in conjunction with other indicators maybe of value in breast cancer prognosis. (Supported by US Army Grant DAMD 17-96-1-6252)

#3001 Disparate p16 and p16β expression patterns in normal and leukemic myelopoiesis. Tschan, M., Vonlanthen, S., Peters, U.R., Oppliger, E., Fey, M.F., and Tobler, A. *Departments of Hematology and Medical Oncology, University and Inselspital, Berne, Switzerland*

Cell cycle control is often disturbed in hematological neoplasia. Disparate ex-pression of $p16^{INK4a}$ and p16β ($p19^{ARF}$) is seen in normal hematopoiesis, and may contribute to leukemiogenesis. Normally p16 inhibits Rb phosphorylation and p16β stops the cell cycle in G_0-G_1, too, but its pathway is not known so far. We measured p16 and p16β expression by semiquantitative reverse transcription PCR (RT-PCR) using two different RNA competitors, by immunocytochemistry and Western blotting. 36 acute myeloblastic leukemias (AML) of different FAB subtypes, 7 samples of $CD34^+$ stem cells and 10 samples of normal monocytes and granulocytes were studied. p16 and p16β expression were detected in all samples, but p16β was higher expressed in leukemic blasts compared to $CD34^+$ stem cells and normal myeloid cells. p16 protein was expressed in parallel to the mRNA transkripts. The p16/p16β ratio was estimated by multiplex RT-PCR and revealed values >1 for $CD34^+$ stem cells and granulocytes, but values ≤1 for most of the AML samples. Methylation of the p16 exon1α was seen in 5/6 AML patients with undetectable p16 protein and mRNA expression but high levels of p16β transkripts. In contrast in 3/3 AML with easily detectable p16 and p16β expression no methylation was observed. In AML p16β expression is distinctly higher than in normal myeloid cells. Absence of p16 in AML is in part due to methylation of p16 exon1α. These findings point to important differences in the expression of these cell cycle parameters in myeloid leukemia, supporting the view that alteration of p16/p16β expression is relevant for leukemiogenesis.

#3002 INK4a exon 1β is wildtype in MCF-7. Van Zee, K. J., Calvano, J. E., Bisogna, M., Borgen, P. I. *Memorial Sloan-Kettering Cancer Center, New York, NY 10021.*

The structure and regulation of INK4a is quite complex. There are two known transcripts of INK4a which code for unrelated proteins. $p16^{INK4A}$ results from transcription of exons 1α, 2, and 3. $p19^{ARF}$ is transcribed from a unique first exon (exon 1β, located 5′ to exon 1α) and exons 2 and 3. $p19^{ARF}$ is translated in an alternate reading frame, creating a completely distinct protein. Because exons 1α and 1β are from the same gene, they are frequently deleted together in many tumor types. This results in difficulty distinguishing the functions of $p16^{INK4A}$ and $p19^{ARF}$. Since MCF-7 has been shown to retain the p15 gene, but have a homozygous deletion of $p16^{INK4A}$, it appears that the breakpoint of the p16 gene deletion is between these two genes. Here we report the presence of wildtype exon 1β in this breast carcinoma cell line. Genomic DNA from MCF-7 was isolated using standard techniques. Exons 1 and 2 of the p15 gene, and exons 1β, 1α, 2, and 3 of the INK4a locus were each amplified by PCR. The products were examined by both agarose gel electrophoresis and SSCP techniques, demonstrating the absence of exons 1α, 2, and 3 of INK4a and the retention of the wildtype exon 1β and the p15 gene. Southern blotting confirmed the homozygous deletion of the p16 gene and sequencing verified the presence of the wildtype exon 1β sequence. Our data demonstrate that the breakpoint for p16 gene deletion in the cell line MCF-7 lies 3′ to exon 1β. Because an intact exon 1β may be sufficient for $p19^{ARF}$ function, this finding suggests that a functional $p19^{ARF}$ may be present even in the complete absence of $p16^{INK4A}$.

#3003 Frequent deregulation of p16 and the G1 cell cycle control pathway in neuroblastoma. Diccianni, M.B., Omura-Minamisawa, M., Batova, A., Bridgeman, L., and Yu, A.L. *University of California at San Diego, San Diego, CA 92103.*

Alterations of the p16 gene in neuroblastoma are very rare. Pronounced expression of p16 at both the transcript and protein level, on the other hand, was observed in 7 of 19 neuroblastoma cell lines (39%) and 2 of 6 primary neuroblastoma samples (33%). As p16 expression is tightly controlled in a feedback loop with pRb, we investigated the possibility that changes in p16 expression were reflective of alterations of the downstream checkpoints in the G1 regulatory pathway. Two cell lines and one primary sample highly expressing p16 were shown to harbor CDK4 amplification. Cyclin D2 was expressed in only three of 20 cell lines, but did not correlate with p16 expression. No significant differences in the expression of CDK6, cyclin D1 or cyclin D3 were evident among any of the cell lines or primary samples, regardless of p16 status. Cyclin E was expressed in multiple isoforms in all neuroblastoma cells lines, but did not correlate with p16 expression. No mutations to the p16 binding site of CDK4 and CDK6, nor to p16 itself were identified in any cell line or primary samples. All cell lines demonstrated an intact pRb protein, yet no correlation with pRb phosphorylation status and p16 expression was observed. Thus, neuroblastoma harbors multiple alterations in the G1 checkpoint, including p16 overexpression. The latter appears to be independent of other G1 phase alterations in the majority of cases and may be indicative of a dysfunctional regulatory control mechanism for p16 yet to be elucidated.

#3004 No evidence for homozygous deletions of the $p15^{INK4B}$ tumor suppressor gene in primary malignant melanoma. Bogenrieder, T., Muehlbauer, M., *Bosserhoff, A.-K., Kroiss, M.M., Landthaler, M., and Stolz, W. *Departments of Dermatology and *Pathology, University of Regensburg, D-93042 Regensburg, Germany.*

Recent studies demonstrate homozygous deletions of $p15^{INK4B}$ in several human primary malignancies (glioblastoma, lymphoma, lung carcinoma), suggesting a role for this gene in carcinogenesis. We therefore examined the deletion status of p15 in malignant melanoma (MM) using a multiplex PCR approach previously employed to assess $p16^{INK4A}$ deletions (Proc. AACR, *37*: 580, 1996). Genomic DNA from 4 MM cell lines with p16 homozygous deletions, 19 sporadic primary invasive (Clark levels II-IV) and 2 metastatic (lymph node) MM specimen (2 of which have p16 deletions) was amplified with primers for p15 exons 1 and 2. The chromosome 9q marker D9S196 served as internal control. We could not identify a homozygous deletion in any of the investigated cell lines or specimens, indicating that, in contrast to p16, p15 is not inactivated by homozygous deletions in primary MM. These data suggest that the inactivation patterns and the respective inactivation mechanisms of these two genes are different in various MM, highlighting the need to search for other candidate tumor suppressor genes on 9p21 as well as other mechanisms of inactivation.

#3005 Inducible p16 expression in MDA-MB-453 breast carcinoma cells. Keller, P.R., and Fry, D.W. *Parke-Davis Pharmaceutical Research 2800 Plymouth Rd, Ann Arbor, MI 48105.*

An inducible p16 expression system has been developed to determine how breast tumors which overexpress cyclin D1 will respond to prolonged suppression of CDK4. The strategy used Lacswitch™ technology in which p16 expression is induced by isopropyl-thio-β-D-galactopyranoside (IPTG). MDA- MB-453 breast carcinoma cells were transformed by a Lac-repressor expressing vector pCMVLacI and clones which strongly expressed the Lac repressor were then transfected with a p16 lac-operator containing vector. From these transfections, 2 clones were identified that highly express p16 in the presence of IPTG as

#1756 Loss of telomerase activity following treatment of testicular cancer cell lines with DNA damaging drugs is not related to apoptosis. Cressey, T.R., Tilby, M.J., and Newell, D.R. *Cancer Research Unit, University of Newcastle-upon-Tyne, Framlington Place, Newcastle-upon-Tyne, NE2 4HH, UK.*

Loss of telomerase activity following treatment with cytotoxic drugs is reported to be associated with the induction of apoptosis (Faranoi et al, Clin. Cancer Res, 3: 579, 1997); however, telomerase loss can be drug specific (Burger et al, Eur. J. Cancer, **26**: 638, 1997). The time courses of telomerase loss (TRAP assay) and induction of apoptosis (nuclear morphology and Annexin V staining) were investigated in two testicular cancer cell lines, Susa CP and 833K, following a 4h exposure to cisplatin, melphalan or doxorubicin. After treatment with $100\times IC_{50}$ concentrations (SRB assay) levels of apoptosis were similar for each drug at each time point, eg. cisplatin, melphalan and doxorubicin caused 64%, 50% and 52% apoptosis, respectively, in Susa CP cells at 24 hours. Telomerase activity 24h after cisplatin treatment was 56% and 33% of control in Susa CP and 833K cells, respectively. However, after doxorubicin there was no significant loss of telomerase activity at 24h or 48h (Susa CP). Ten hours after cisplatin treatment in Susa CP and 833K cells telomerase activity was 132% and 103% of control, respectively, whilst extensive apoptosis was already apparent. This investigation shows that, for similar levels of apoptosis, loss of telomerase activity was compound specific. The time courses of telomerase loss and induction of apoptosis were independent in both of the cell lines studied.

#1757 Telomerase and clinicopathologic prognostic factors in early stage cervical cancer. Wisman, G.B.A., Knol, A.J., Helder, M.N., De Vries, E.G.E., Hollema H., De Jong S., Van der Zee, A.G.J. *Depts. of Gynecology and Medical Oncology, University Hospital Groningen, The Netherlands.*

Telomerase activity (TA) is frequently upregulated in cervical cancer. However, little is known about the possible applicability of telomerase as a prognostic factor. Aim: To examine the relation between telomerase activity (TA), telomerase RNA (hTR) and/or catalylic subunit (hTERT) and known clinicopathologic prognostic factors in cervical cancer. Methods: Frozen specimens were obtained from 54 patients with stage I/II cervical cancer, primarily treated with surgery. Clinicopathologic and follow-up data were stored in a dbase (median follow-up 30 mo(24–61). TA was determined with fluorescence-based TRAP, hTR and hTERT with semi-quantitative RT-PCR. Results: TA levels were related to hTERT ($r=0.6$, $p<0.02$), but not to hTR expression. Frequency of TA was related to differentiation grade (11/15 (73%) grade I, 14/18 (78%) grade II and 14/14 grade III ($p<0.05$), but not to stage (19/27 (70%) stage Ib1, 9/11 (82%) Ib2 and 13/16 (81%) IIa), histotype (squamous cell 30/37 (81%) vs. adeno 10/14 (71%) or presence of locoregional lymph node metastases (24/34 (77%) in node negative vs. 12/13 (92%) in node positive patients. Sofar, no relation with locoregional recurrence ($n=4$) or (progression free) survival could be established, but the number of events is small. Conclusion: TA in cervical cancer appears to be mainly regulated by hTERT levels and is related to differentiation grade. In order to evaluate more reliably the value of telomerase expression as prognostic factor an extended series of patients will be analyzed and presented. *Supported by grant NKB/KWF: 97–1581.*

#1758 Telomerase RNA expression and cell proliferation index in vulvar intraepithelial neoplasia (VIN) and carcinoma. Rathi A, Flowers L, Virmani AK, Ashfaq R, James Scurry[1], Muller C, Gazdar AF. *UT Southwestern Medical Center Dallas, TX. and [1]Mercy Hospital for Women, Melbourne, Australia.*

Deregulation of telomerase is associated with tumerogenesis and provides unlimited proliferation capacity for cancer cells. We examined the in situ expression of telomerase RNA as well as cell proliferation activity determined (by Ki 67 expression) by reactivity with MIB-1 antibody during the step-wise progression of vulvar cancer. Paraffin embedded tissue from 20 cases representing 19 invasive squamous cell carcinomas (9 HPV positive, 10 HPV negative) and one case of VIN III without adjacent tumor, 42 associated normal and dysplastic mucosal foci and 7 biopsies (as control) from subjects without vulva cancer were analyzed. The pattern of hTR expression varied with the grade of the lesions and showed a moderate correlation with cell proliferation index (Kendall's tau-b = 0.423). Both hTR expression and MIB-1 increased significantly ($p=0.001$) with the histological grade (VIN I to III). In contrast to MIB-1 index which showed a wide range, hTR upregulation occurred in the majority of advanced precursor lesions associated with invasive tumor irrespective of the HPV status. Upregulation of hTR expression in VIN III may be useful as a biomarker for risk assessment.

#1759 Identification of the human mammary epithelial cell subpopulation expressing telomerase. Bednarek, A.K., Brenner, A., and Aldaz, C.M. *Department of Carcinogenesis, University of Texas M.D. Anderson Cancer Center, Science Park- Research Division, Smithville, TX 78957.*

Most immortal and tumor cells express telomerase. It has been speculated that expression of this enzyme is necessary to escape cellular senescence. More recently however, this enzyme was found to be normally expressed in adult epithelial tissues, in association with the subcompartment that contains cells with self-renewing potential. Experiments were performed to determine which cell subpopulation from the human mammary gland epithelium normally express this ribonucleoprotein. Samples were obtain from normal human breast mammoplasty specimens. Cells from the luminal and myoepithelial compartments were isolated after enzymatic dissociation of fresh mammary epithelial organoids. Sorting of the two different epithelial cell types (luminal or myoepithelial) was performed by immunodetection of specific membrane antigens and absorption to immunomagnetic beads. Epithelial membrane antigen (EMA) was used to isolate the luminal cells and CALA to sort the myoepithelial cells. We used the telomeric repeat amplification protocol (TRAP) to demonstrate telomerase activity. We observed that EMA positive cells (luminal) showed telomerase activity while the CALA positive cells (myoepithelial) were telomerase negative. This analysis indicates that the myoepithelial cells may belong to a terminally differentiated compartment while the cells with proliferative (self-renewing) potential belonged to the luminal compartment. (Supported in part by US Army Grant DAMD 17-96-1-6252)

#1760 Down-regulation of telomerase activity and hTERT mRNA expression by autocrine transforming growth factor β. Yang, H. and Sun, L.-Z. *Dept. of Pharmacology, University of Kentucky College of Medicine, Lexington, KY 40536.*

Telomerase activity is associated with cell proliferative potential, transformation and malignant progression. Transforming growth factor β (TGFβ) suppresses epithelial cell proliferation. Loss of autocrine TGFβ growth inhibitory activity is a hallmark of solid tumor progression. To test our hypothesis that autocrine TGFβ may negatively regulate telomerase activity, we have compared telomerase activity and human telomerase reverse transcriptase (hTERT) mRNA levels in two pairs of human cancer cell lines whose autocrine TGFβ activity was either enhanced or suppressed. HCT116 colon cancer cells do not have autocrine TGFβ activity due to the mutation of its type II receptor (RII). Re-expression of RII restored autocrine TGFβ activity. This also led to a significant reduction of the telomerase activity measured with the telomeric repeat amplification protocol (TRAP) and the hTERT mRNA level. On the other hand, suppression of the autocrine TGFβ activity with a kinase-defective dominant-negative RII in MCF-7 breast cancer cells led to a significant increase of telomerase activity and the hTERT mRNA level. Flow cytometry analyses indicated that the regulation of telomerase activity by autocrine TGFβ was independent of the population of the cells in various cell cycle stages. Our observations suggest that autocrine TGFβ may be an important regulator of telomerase activity in human cells.

#1761 Application of kinetic PCR, hTR in situ hybridization, and hTERT immunoassays to the detection and measurement of telomerase expression in cells and tissues. Kim, N.W., Amshey, S., Chang, S-Y, Emrich, T., Santini, C., Spaulding, D., Cass, L., and Weinrich, S.W. *Geron Corporation, Menlo Park, CA 95129, Roche Molecular Systems, Alameda, CA 94501, DAKO Corporation, Carpinteria, CA 93013, and Roche Molecular Biochemicals, Penzberg, Germany.*

Telomerase activity is specifically associated with cancer and immortal cells and is found in all cancer types that have been examined to date. Cloning of the RNA (hTR) and the catalytic protein (hTERT) components of human telomerase provide alternative means for measuring the expression of telomerase in cells and tissue samples. Additionally, application of recent advances in quantitative PCR and signal amplification technologies provides efficient, reliable, and accurate telomerase assays that significantly improve the analysis of clinical samples for telomerase expression. We have developed kinetic PCR-based TRAP, hTR RT-PCR, and hTERT mRNA RT-PCR assays using the LightCycler™ that can accurately perform each of these assays in 30 min. Analysis of various cancer and immortal cell lines indicated that steady state level of hTR and hTERT mRNA, based on rRNA levels, are approximately 400 and 4 copies per cell, respectively. We have also developed immunohistochemistry and ELISA using monoclonal antibodies against hTERT polypeptide fragments. The hTERT immunohistochemical assay has been used in parallel with a non-radioactive hTR in situ hybridization assay to study the intracellular localization of hTR and hTERT in immortal cells. We have also used these assays to measure levels of the telomerase components in tissue and fluid samples. The results from these analyses support clinical applications of telomerase in diagnosis, monitoring, and screening of cancers.

#1762 Telomerase activity in adriamycin resistant-human breast carcinoma MCF-7 cells. Ishikawa T, Kamiyama M, Hisatomi H, Ichikawa Y, Momiyama N, Hamaguchi Y, Hasegawa S, Akimoto H, Shimada H. *Yokohama City University. 2nd Dept. of Surgery, Yokohama, Japan 236-0004.*

Decline in telomerase activity has been reported in cancer cells after treatment with anti-neoplastic agents. Assessment of telomerase activity could be a valuable tool to measure the reduction of aggression due to chemotherapy. This study was designed to investigate the significance of telomerase for chemotherapy with respect to adriamycin (ADM)-resistance. **METHODS** MCF-7 and its ADM-resistant line (AdrR) were treated with ADM, 5-fluorouracil (5FU) or Taxotere (TAXO). Telomerase activity and human telomerase RNA component (hTR) were quantitatively measured by the telomeric repeat amplification protocol assay and RT-PCR respectively. Cell number counting and MTT assay were also performed. **RESULTS** In MCF-7, enzyme activity was significantly reduced by ADM, 5FU or TAXO treatments (55 ± 18, 60 ± 19, 65 ± 24% of control, mean ± SE). In AdrR, 5FU and TAXO reduced enzyme activity (68 ± 17%, 70 ± 21%), while ADM significantly increased the activity (135 ± 30%). No significant changes in hTR were seen in these 2 cell lines after treatment of any of these drugs. When Bcl-2 expression was examined after drug treatments by Western blot, ADM increased Bcl-2 expression in AdrR cells, while slightly decreased in MCF-7 cells. **CON-**